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DEGRADATION OF WASTE PAPER TO PROTEIN. RESEARCH IN MICROBIAL FERMENTATIONS

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Research in Microbial Fermentations

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DEGRADATION OF WASTE PAPER TO PROTEIN

Research in Microbial Fermentations

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ABSTRACT

The early stages of this work concentrated on efforts to isolate pure cultures which would utilise both cellulose and hydrocarbons. From more than 70 different inocula of soil, compost, sewage sludge and forest litter, 367 pure cultures were isolated on n-hexadecane media; 28 of these were found to be capable of attacking sodium carboxymethyl cellulose and lowering its viscosity. Only one proved to be capable of attacking purified cellulose, ball-milled newspaper, or newsprint. This culture is a fungus which grows well up to 45 C, identified as Aspergillus fumigatus.

More than 300 enrichment cultures were also developed on cellulosic substrates (purified cellulose, ball-milled newsprint, without ink, or ball-milled newspaper) from similar inocula. These were tested in shake flask fermentations for ability to produce protein and utilize the cellulosic substrates listed above. Only 10 enrichment cultures yielded greater than 0.2 mg of protein per ml in 5 days. These also utilized appreciable amounts of cellulose. From the active enrichment cultures, 5 cellulolytic pure cultures were isolated. All of these were fungi. All gave considerably higher protein yields and cellulose consumption under shake flask conditions than the known cellulolytic organisms Trichoderma viride or Cellulomonas sp. These organisms were studied in shake flask fermentors in an effort to achieve optimum rates of cellulose utilization and protein synthesis. The final identification of the five fungus cultures was made by Dr. Richard N. Kinsley, Jr., for which we express our gratitude and appreciation. Two of the cultures were Myrothecium verrucaria, two were Aspergillus fumigatus and one was Trichoderma lignorum.

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Myrothecium verrucaria gave the highest rates of protein synthesis of any of the fungi studied in shake flask studies; therefore, follow-up studies using stirred-jar fermentors concentrated on this organism (culture P9). Conditions were optimized during 12 runs in 14-liter stirred-jar fermentors. Protein yield on ball-milled newspaper increased with substrate concentration up to the maximum used. It was shown that a very simple medium containing dibasic ammonium phosphate, 1%, urea, 0.03%, and yeast autolysate, 0.1%, was optimal for growth and protein synthesis on ball-milled newspaper, 4 g per 100 ml of medium. Under these conditions (run 11) the maximum rate of cellulose consumption was 5.4 g/l/day, and the rate of protein synthesis was 0.3 g/l/day. The maximum yield of protein obtained was 1.42 g/l by the highly specific modified biuret method, or 3.3 g/l by usual method of multiplying total organic (Kjeldahl) nitrogen by 6.25. The amount of cellulose consumed in this experiment was 12.7 g/l from an original 20.4 g/l contained in 40 g/l of ball-milled newspaper.

The final product was recovered by evaporation of the culture fluid. Chemical analysis indicates that it may be a nutritious animal feed, but this must be proved by the construction of the pilot plant to manufacture sufficient material for extensive animal feeding studies to evaluate toxicity and nutrient value.

Background Information

Cellulose is perhaps the most abundant of all organic materials in nature, as it is the major structural material of most plants. It sometimes occurs in almost pure state, as in cotton fibers, but more often in association with other compounds. Wood, for example, is a complex of cellulose (40 to 80%), lignin (20 to 30%), hemicelluloses and xylosans (10 to 30%).[1] The lignocellulose complex of softwood is attacked only with great difficulty by cellulolytic enzymes. Thus, paper made from "mechanical pulp," such as newspaper which is simply ground-up softwood, is relatively resistant to degradation by microorganisms. Paper made from chemical pulp, is much more readily attacked, as the hot sulfurous acid or alkaline sulfates used in the chemical treatment have partially delignified the wood pulp.[2] Accordingly, we have chosen to carry out most of our work on newspaper, since any other kind of paper will probably be more easily degraded.

Reese, Siu and Levinson[20] showed that many fungi and bacteria are able to hydrolyze modified cellulose, such as sodium carboxymethyl cellulose, but few are able to attack native cellulose such as cotton fibers. From these and other data they developed a general theory of the dual nature of cellulase enzyme systems. According to this theory, true cellulolytic organisms, those capable of attacking native cellulose, produce two or more enzymes. The first, called C_1, attacks native cellulose to break up the aggregates and produce linear chains of anhydroglucose units. These chains are then attacked by a second enzyme C_x, α β 1,4-glucanase, which hydrolyzes them to the disaccharide cellobiose. Cellobiose may then be assimilated directly into the cell, or may be converted to glucose by β-glucosidase before assimilation. Mandels and Reese[21] have presented the following schematic description of their theory:

cellulose	→	reactive cellulose (linear chains)	→	cellobiose	→	glucose
↑ C_1 | unknown mechanism			↑ C_x | hydrolytic		↑ β-glucosidase | hydrolytic	

These authors also found that woody materials, such as newspaper, were relatively resistant to cellulases unless they were thoroughly ground by ball-milling. They also succeeded in separating the C_1 and C_x enzymes from Trichoderma viride filtrates on columns of DEAE dextran. Since several other authors had questioned the C_1, C_x theory of the dual nature of cellulase enzyme systems, this represented an important advance. The separation of C_1 and C_x activity, and the demonstration that the combination of the two separated enzymes, C_1 and C_x, was necessary to produce soluble sugars from cotton was also achieved by Wood[22] using filtrates of Trichoderma koningii.

Degradation of Waste Paper to Protein

Introduction

In April, 1968, Dr. J. D. Douros prepared a detailed progress report covering the progress of the project up to that time; on December 30, 1968 and February 28, 1969 other progress reports were submitted by the writer. Since all those who read this final report may not have seen the earlier reports, they shall be summarized here.

The primary emphasis in the earlier stages was placed on attempts to discover microorganisms which would utilize both cellulose and hydrocarbons. Most of the cultures were isolated on hydrocarbon media, and their ability to utilize cellulose was not evaluated until later. Many other cultures were isolated on media containing both cellulose and hydrocarbons. In the earlier work, up to August 1968, 60 different samples of soil and decaying wood and vegetation were obtained from different areas, some near paper mills, saw mills, petroleum seeps, compost piles, and forest litters. Also a lyophilized microbial preparation sold by Reliance Chemical Co. for the degradation of cellulosic wastes was evaluated, as well as sewage sludge samples.

Methods

The hydrocarbon-oxidizing organisms (337 cultures) were isolated from the inocula of soil, compost, sludge, etc. by both liquid enrichment in one of the seven media listed in Table I of the Appendix supplemented with 1% of n-hexadecane, or by the sprinkle plate technique, wherein the inoculum is sprinkled over the surface of the hydrocarbon medium solidified with 2% agar. Also more than 300 enrichment cultures were developed on many different media contained in shake flasks, using cellulose, newsprint (no ink) or newspaper as the major or sole energy source. The enrichment cultures were streaked out on agar plates containing the isolation substrate, in the case of hydrocarbons, or regenerated cellulose agar[7] for cellulose or paper-utilizing cultures, for pure culture isolation. The streaking and isolation step was repeated, and a Gram stain was made. If visually pure, a fermentation was carried out for 24 hours under shake flask conditions resembling the isolation treatment. At 24 hours, a Gram stain was again done, and visually checked for purity. If pure, technicians prepared a permanent slide and froze three tubes of the original fermentation broth, and inoculated litmus milk for a second culture to deep freeze. Cultures for transfer purposes were grown on mineral salts agar + the isolation substrate. Nutrient agar slants for fermentation inocula were inoculated from the mineral salts agar slants in order not to attenuate the culture.

The earlier work was impeded by the lack of a specific analytical method for cellulose, so that it was not possible, in most cases, to determine whether cellulose was being consumed, or whether protein was being produced from other nutrients in the culture medium, such as peptone, yeast extract or hydrocarbons.

During the period from August to December 1968, accurate analytical methods were developed for total protein (a modification of the biuret method)[3] and for cellulose. The latter is a semi-micro modification of the classical gravimetric method of Crampton and Maynard (1938)[4] in which lignin, hemicelluloses and other carbohydrates are extracted with acetic acid-nitric acid reagent. The purified cellulose which remains is dissolved in 67% H_2SO_4, and determined by the anthrone reagent.[5] The method has recently been published[6].

The preliminary screening of cultures for cellulolytic ability was carried out by the usual methods: (1) Inoculation into Cellvibrio medium containing a strip of Whatman No. 1 filter paper. Cellulolytic activity was indicated by digestion of the paper[7]. (2) Streaking on plates of regenerated cellulose agar: Cellulolytic activity was indicated by the formation of clear zones surrounding the colonies[8]. (3) Inoculation into CMC medium: Cellulolytic activity was indicated by liquifaction of the very viscous CMC Medium[9]. (4) Assaying the culture for cellulase activity on sodium carboxymethyl cellulose (CMC, Hercules Co., type 7 MF) by a filter paper disc diffusion assay using E. coli or P. aeruginosa as assay organisms to detect the glucose produced by action of the original culture on cellulose or CMC. This assay procedure was described in detail in Dr. Douros's last progress report. Since it has not yielded reliable predictions of good cellulolytic activity, it has since been abandoned in favor of the more accurate biuret and cellulose analyses.

All cultures giving any evidence of cellulolytic activity by any of these procedures were then studied in shake-flask fermentations. Most of them were grown on three different cellulosic substrates, and in two or three different culture media. The cellulosic products employed were purified cellulose, Nutritional Biochemicals Co., ball-milled newspaper, and ball-milled newsprint (no ink) obtained from the Denver Post. The composition of the culture media employed are given in the appendix to this report. In the earlier part of the study, the final fermentation liquors were homogenized and analyzed both for protein by the biuret method and for cellulose consumed by the method described earlier. More recently, since it was found that cultures yielding more than 0.2 mg/ml of protein (on non-hydrocarbon media) always utilized some cellulose, the protein analysis alone has been employed as a screening device for the selection of promising cultures to follow up. The general method employed in the shake flask experiments is described below:

A one-gram portion of the air dried cellulosic substrate was weighed into a 500 ml baffled shake flask, and 100 ml of the desired liquid medium was added. The flasks were plugged with polystyrene foam plugs, and were autoclaved for 20 minutes at 20 psig. The flask was inoculated from a source culture using at least 10^8 cells in the inoculum, and flasks were incubated for 2 days on a shaker-incubator at the selected temperature. One ml of this inoculum culture was then employed to inoculate a second flask of the same culture medium. The cultures were incubated for 5 days on a rotary shaker incubator at 350 revolutions per minute. Analyses carried out on each culture included cell count, either by plating or direct microscopic examination, pH, and total protein by biuret. Some cultures were also assayed for cellulase and some were analyzed for the amount of cellulose consumed as described. Where cultures did not appear homogenous, they were treated on a Virtis homogenizer before analysis. The analytical results were corrected for values determined on identical sterile control flasks. Biuret analysis of sterile controls gave small "protein" values

due to colored material in the paper substrate, or in the case of Reese's medium, to the proteose peptone supplement. These values were subtracted from the values given by the cultures to obtain "corrected" values for protein. The "percent of cellulose utilized" figure was calculated by subtracting the amount of cellulose found in the culture from that in the sterile control.

The data on all enrichment cultures which gave more than 0.2 mg/ml protein yield are summarized in Table I. These represent the best-yielding cultures out of many hundreds. The data on the pure culture fermentations yielding more than 0.2 mg/ml of protein are given in Table II. The sources and a brief description of the active cultures are given in Table III.

A study of Tables I, II and III reveals that organisms giving a yield of 0.2 mg/ml or more of protein on purified cellulose or paper in 5 days are not common. Out of the 367 hydrocarbon-utilizing cultures, only one (C264) utilized the cellulosic substrate appreciably, although 21 of these cultures did utilize sodium carboxymethyl cellulose, and 28 of them reduced the viscosity of CMC in the presence of n-hexadecane. Other conclusions from these experiments are: Only 10 promising enrichment cultures were found out of more than 500 tested. From these and other inocula, a total of 5 promising pure cultures were isolated, all fungi. Three of the pure cultures did well on all 3 cellulosic substrates (cultures C264, 353x85-1, & 353x99-B). Cultures P-9 and P-10, showed preferences among the substrates. Both gave better yields on newsprint and newspaper than on purified cellulose. These pure cultures did as well as the enrichment cultures from which they were isolated in decomposing cellulose.

The five pure cultures of cellulolytic fungi described in Table III were inoculated on plates of Sabouraud's agar and Czapek's agar, and incubated for 30 days at room temperature. Macroscopic and microscopic examinations were made at intervals in an effort to identify the organisms. The characteristics of the cultures indicated that we were dealing with 3 species of fungi; we were unable to identify cultures P9 and P10, culture 353 x 99B was tentatively identified as Trichoderma lignorum, and cultures 353 x 85-1 and C 264 were tentatively identified as Aspergillus fumigatus. These cultures were turned over to Dr. Richard N. Kinsley, Jr., Associate Professor of Microbiology at East Tennessee State University, who provided the final identifications given in Table III, for which we express our gratitude and appreciation.

Unfortunately Aspergillus fumigatus is a known pathogen, being the cause of Aspergillosis, a pneumonitis of birds, which also occasionally infects humans[10]. Therefore cultivation on a larger scale was not believed to be advisable.

Optimization of Growth Rate of Pure Fungus Cultures

The five pure cultures described above were grown under different conditions of time, temperature, and medium composition in 500 ml shake flasks containing 1 g of ball-milled newsprint and 100 ml of medium with shaking at 350 RPM. Direct microscopic examinations, pH measurements,

protein analyses by the biuret method, and cellulose analyses were made at intervals of 2, 5 and 9 days. The data are given in Table IV.

The conclusions drawn from a study of Table IV are summarized below:

1. P-9 and P-10 grow well at 30 C and 37 C. Protein synthesis is most rapid at 30 C, but cellulose utilization is greater at 37 C. These cultures grow very poorly or not at all at 45 C.

2. 353 x 85-1 and C264 grow well at all three temperatures. Protein synthesis is more rapid at 37 C and 45 C than at 30 C. Cellulose utilization is also greater at 37 C and 45 C.

3. 353 x 99B grows, synthesizes protein, and utilizes cellulose well at 30 C, very poorly at 37 C and not at all at 45 C. Culture No. 72 appears to be an exception to this, but it is believed to have been contaminated with 353 x 85-1 or C-264.

4. P-9, P-10 and 353 x 99B synthesize slightly larger amounts of protein in 5 or 9 days than 353 x 85-1 or C-264. The percentage of cellulose utilized was not significantly different among the different cultures for those flasks giving high protein yields.

5. All actively growing cultures produce acid, as shown by decreasing pH, but P-9 and P-10 produce slightly more than the other cultures.

6. All of the cultures are very actively cellulolytic, and utilize more than 80% of the cellulose in 9 days under optimum conditions.

7. TV medium is superior to SM for P-9 and P-10, the two media are equal for 353 x 85-1 and C-264, and SM is slightly superior for 353 x 99B for protein synthesis. However, TV is slightly superior for cellulose utilization by 353 x 99B.

Two remaining aspects of optimization, paper concentration and medium simplification, were then investigated in two additional experiments.

All other factors being equal, the rate of biosynthesis of cell protein should increase with increasing substrate concentration. A preliminary experiment was made with ball-milled newsprint (no ink) and ball-milled newspaper. With both materials, it was found possible to disperse up to 30 g of paper in 100 ml of aqueous medium without making the system too viscous for agitation on a shaker. On the other hand, when newspaper was pulped in a Waring blendor with aqueous medium, a maximum of 2 g of paper could be dispersed in 100 ml of medium before the system became too viscous to shake. Microscopic examination revealed the reason. The blended newspaper consisted mostly of long fibers, whereas the fibers were relatively short in the ball-milled paper.

A preliminary experiment was set up using cultures P9, P10, and P-26 to evaluate comparative growth on different concentrations of ball-milled newsprint, newspaper, purified cellulose and blended newspaper in Reese's

medium. The evaluation of growth was carried out by direct phase contrast microscopic examination of samples from the shake flasks after 2 days' and 5 days' growth.

The data, summarized in Table V, indicate that fair to good growth may be obtained on any concentration of any of these cellulosic substrates. There appears to be some degree of growth inhibition at the highest concentration of paper, but since the effect is slight, it could probably be overcome by adaptation of the inoculum cultures.

Another objective of these experiments is to develop the lowest cost process possible. This necessitates the use of simple treatment methods and cheap reagents. Chemicals for media should be low cost fertilizer grade, and ordinary tap water should be used in place of distilled water. Some prices, selected from the Oil Paint and Drug Reporter are given below:

	$/Ton	$/Ton of N
1. $(NH_4)_2SO_2$ (fert. grade, 21% N)	33	157
2. NH_4NO_3 (33.5%N)	45	134
3. $(NH_4)_2HPO_4$ (tech. grade, 15% N & 45% P_2O_5)	84	466
4. Urea (fert. grade, 46% N)	92	199
5. Anhydrous ammonia (fert. grade) 82.5% N	50	61
6. Phosphoric acid (H_3PO_4, fert. grade) 52-54% available phosphoric acid	53	-
7. $(NH_4)_2HPO_4$ (fert. grade, made by reacting 5 (anhydrous ammonia) and 6(phosphoric acid)	52	348

These chemicals are available in car-load lots at prices far below individually packaged chemicals. Thus it would probably be advantageous to use these crude materials wherever possible, blending them to obtain as many of the essential mineral nutrients as possible. Those mineral nutrients not supplied in the mixture would probably be supplied in adequate amounts from the paper and tap water used.

The major nutrients not supplied in adequate quantities by the waste paper and tap water are nitrogen and phosphorous. Nitrogen could be obtained from either ammonia, urea, or any of the ammonium salts listed. Phosphate could be obtained from phosphoric acid, or from ammonium phosphate. It is clear from the table that nitrogen could be obtained most cheaply from fertilizer grade anhydrous ammonia, and phosphate from fertilizer grade phosphoric acid.

In order to evaluate these factors, culture media were prepared using 1% fertilizer grade $(NH_4)_2SO_2$ and 1% reagent grade $(NH_4)_2HPO_4$ (since fertilizer grade was not available at the time), supplemented with 2% ball-milled newspaper. Flasks containing the same substrates in Srinivasan's medium were included for comparison. Shake flasks were set up as previously described, inoculated with 5 day old cultures of the same 3 test fungi, and incubated at 30°C. Cultures were harvested at intervals of 2, 5 and 9 days, and analyzed for protein; pH was also measured as described earlier.

The data are presented in Table VI. The conclusions derived from these data are that growth of all 3 fungi is slightly more rapid in Srinivasan's medium during the early growth phase, as shown by a higher protein yield in 2 days, but yields in 5 and 9 days were far superior on the ammonium phosphate medium (G). Yields on the ammonium sulfate medium (F) were approximately equal to those on Srinivasan's medium. Considering that Srinivasan's medium contains 6 different salts, and yeast extract, the superiority of medium G, containing only a single salt in tap water is remarkable.

As a follow up on the last experiment with media F and G, prepared with tap water and ammonium sulfate and tap water and ammonium phosphate, an experiment was set up with medium H, containing equal parts of the two salts. The other conditions were the same except that the inoculum cultures were taken from the preceding experiment in medium G, and were 16 days old, having been stored in the refrigerator for a week after the 9 days' incubation.

The data are summarized in Table VII. The data indicate lower rates of protein synthesis, as well as lower final protein yields, than for the preceding experiment. Medium H appears to be approximately as good as medium F, but inferior to medium G. The slower rate of protein synthesis in this experiment may have been caused by the difference in inoculum, that is the longer incubation time or the lack of an organic nutritional supplement in the medium. The inoculum for the preceding experiment was grown in Srinivasan's medium, which is supplemented with yeast extract, whereas medium G contains no such supplement. Microscopic examinations revealed very heavy conidiospore formation by Myrothecium verrucaria in Srinivasan's medium and Reese's medium, but little or no conidiospore formation in medium G.

Cellulomonas

An experiment in which a culture of Cellulomonas sp., obtained from Dr. V. R. Srinivasan of L.S.U., was compared with 4 of our bacterial isolates closely resembling Srinivasan's culture in cultural characteristics morphology, and certain biochemical reactions, is summarized in Tables VIII and IX. Table VIII indicates that all 5 cultures had similar morphology, and all grew well in both media and digested filter paper and CMC at similar rates. All were small Gram-variable rods, non-motile, methyl red negative, Voges-Proskauer negative, indole negative and reduced nitrate to nitrite. The oxidation-fermentation test showed a negative reaction on glucose, but slight fermentation by some on starch, cellobiose, sucrose or lactose. Table IX shows that all grew in the fermentation media on paper and purified cellulose, but produced very low protein yields in 5 days. All of the isolates are probably Cellulomonas, although not necessarily the same species. Since they did not produce good protein yields on newspaper, no further studies were made on these bacteria.

A culture of an actively cellulolytic strain of Trichoderma viride (QM No. 6a) was obtained from Dr. T. K. Ghose of the U.S. Army Laboratories, Natick, Mass., and grown on Reese's medium with 1% newsprint and Srinivasan's

medium with 1% newsprint. The data, presented in Table X indicate extensive utilization of cellulose, but poor protein yield at 37°C, but good protein yield at 30°C. The yields on this fermentation, carried out under the same conditions as those of Table IV were closely comparable with those obtained with P9 and P10 (Myrothecium verrucaria) and P26 (Trichoderma lignorum).

Chemical Treatment of Paper

Newspaper (Wall Street Journal) was pulped in a Waring Blendor with 500 ml of distilled water for each 10 g of paper. The paper was filtered out and dried in an oven at 105 C. After cooling at ambient conditions, each portion was weighed and treated with 1 M solutions of the test reagents, NaOH, H_2SO_4, H_3PO_4 and $NaHSO_3$. One series was stirred with the reagent at room temperature for 8 hours, and allowed to soak for 4 days. Samples were then filtered out, washed with tap water until washings were neutral, and dried and weighed as before. Another series was heated in a steam autoclave at 19 psi for one hour, allowed to soak for four days, filtered out, washed and weighed. The data are given in Table XI.

The chemically treated paper samples were then dispersed in medium H and fermented in the usual manner along with other samples containing ball-milled paper. The inoculum was taken from the experiment described in Table XI, and was grown in medium H for 5 days. The data are given in Table XII. Protein yields were disappointingly low for all samples, even those grown on ball-milled paper, probably reflecting a poor state of the inoculum. Chemically treated paper gave good growth, as indicated by microscopic examinations, but protein yields were below those from ball-milled paper in nearly all cases. None of the chemically treated papers appeared to give protein yields superior to paper treated with plain water.

Effect of Growth Medium on Inoculum

An experiment was then set up to determine whether protein production is superior from flasks inoculated from a vegetative seed grown on Reese's medium or on medium G. Microscopic examination of the inoculum cultures after 2 days' incubation revealed much heavier growth in Reese's medium, and the presence of a large number of conidiospores in Reese's medium only. The experiment also compared medium G with medium H. The 5-day protein yields (see Table XIII) were far superior from harvest flasks inoculated with cultures grown in Reese's medium. Cultures P9 and P10 gave essentially equal protein yields on medium G and H, but P26 gave a better yield on medium G. The protein yields from P9 and P10 were superior to those from P26.

Cellulose Synthesis by Fungi

An experiment was then set up to determine the amount of cellulose synthesized by fungi P9, P10, P26 and C264 grown on 10% glucose in Reese's medium. The data, given in Table XIV, indicate that P9 and P10 contain no cellulose, while P26 and C264 contain considerable cellulose. P9 and P10 synthesized more protein then P26 or C264.

Ball-milled vs. Dry-chopped Paper as a Substrate

An experiment was carried out to compare protein yields from P9 and C264 grown on Medium H with 2% newspaper chopped dry in a Waring Blendor vs. 2% ball-milled newspaper. The data, summarized in Table XV indicate considerably higher 5-day yields from ball-milled newspaper.

Effect of pH, Buffers and Trace Elements

An experiment was set up sto determine the effect of pH, trace elements and buffering by means of $CaCO_3$ on cellulose utilization by fungi growing on newspaper in Medium G. The media contained 0.005% bromcresol green in order to permit visual pH determination. The pH values of flasks 21-30 were adjusted on the third, fourth and fifth days of incubation by dropwise addition of sterile 0.1 N NaOH. Biuret analyses of protein could not be done because of interference from the indicator dye, but cellulose utilization was determined on all flasks. The data, given in Table XVI, indicate slightly higher cellulose utilization in the absence of the trace-elements solution than in its presence, and higher cellulose utilization in those cultures maintained at pH 5.6 to 5.8 by daily titration than in those buffered with $CaCO_3$ (terminal pH 4.9 to 5.3) or those allowed to seek their own pH level (initial pH 4.8 to 5.3, terminal pH 3.1 to 3.9).

Effect of Trace Elements Proteose Peptone and Urea

An experiment was performed to determine the effect of added trace elements, urea and proteose peptone on protein yield and cellulose utilization by fungi growing on newspaper in medium G. The interactions of these variables were studied in both inoculum and harvest cultures. The 5-day harvest data (see Table XVII) on protein yields and cellulose utilization indicated no consistent effect of trace elements, a slight stimulatory effect on protein synthesis by peptone, and definite stimulatory effect from urea on both protein yield and cellulose utilization. The flasks containing urea also had higher pH values (from 4.4 to 5.9 vs. 3.4 to 4.2 in flasks without urea). This, taken with the results of previous experiments, suggests that urea may act merely by keeping the pH in the optimum range. Assuming that the fungi produce urease, the hydrolysis of one mole of urea to 2 moles of ammonia and one mole of carbon dioxide would decidedly increase pH.

A follow-up on this experiment explored the interaction of these variables in other combinations, and compared 2-day growth and protein yield from vegetative seed cultures grown on Reese's medium and medium G supplemented with urea, urea and trace elements, and urea, trace elements and protopeptone. Data are given in Table XVIII. Growth and protein yield in two days was by far the best in Reese's medium. Again, urea appeared to increase yield markedly, while the trace elements and protopeptone had little effect.

Earlier experiments showed that Reese's medium gives more rapid growth than medium G, even when medium G was supplemented with trace elements,

urea and protopeptone, but medium G gave higher protein yields after five days. Microscopic examination showed that Reese's medium caused cultures P9 and P10 to produce very large numbers of conidiospores as well as some mycelium, whereas only the mycelial form was produced in medium G. Correlated with this, Reese's medium gave far better results for the vegetative seed than medium G. An experiment was therefore initiated to study the effect of substituting other materials for protopeptone in Reese's medium and Medium G. The data, given in Table XIX, show that casamino acids, is as good as protopeptone in promoting growth and protein synthesis in Reese's medium.

Another experiment was then set up with a modified Reese's medium, in which casamino acids was substituted for protepeptone. The experiment is summarized in Table XX. The data show that the modified Reese's medium gives equal or better protein yields when made up with tap water than when made up with distilled water. Surprisingly, the best protein yields were obtained from this modified medium from which the $MgSO_4 \cdot 7\ H_2O$, $CaCl_2$ and trace elements had been omitted, showing that sufficient Mg, Ca, and trace-element minerals were available from the tap water and the paper, and in such proportions as to give even better results. Again it was shown that the addition of urea gave a good improvement in protein yield, while the addition of casamino acids gave a lesser improvements.

Effect of Type of Newspaper

Up to this time most of our work was done with ball-milled Wall Street Journal. Since the Denver Post became available to us in shredded form, we carried out an experiment to compare the yield on these two ball-milled materials, using fungi P9, P10 and P26 and Medium G supplemented with trace elements and urea. The data are given in Table XXI. The 2-day vegetative seed media (Modified Reese's with casamino acids) gave higher protein yields from the Wall Street Journal, but the 5-day harvest flasks gave slightly higher yields from P9 and P10 on the Denver Post, but from P26 on the Wall Street Journal.

Effect of Heating Newspaper

Since Ghose[12] has found that heating cellulose to 200 C for 20 minutes gives a substrate providing improved cellulase synthesis by Trichoderma viride, we tried the process on newspaper (Wall Street Journal). After heating and ball-milling the paper was incorporated into medium G supplemented with trace elements and urea, and fermented with fungus cultures P9, P10, and P26. As indicated in Table XXII, the three fungus cultures all gave slightly higher protein yields on the heated paper.

The experimental work carried out thus far indicates that Reese's medium, prepared either with protopeptone or casamino acids as a nutrient supplement, is superior for growing the vegetative seed. In the case of cultures P9 and P10, protopeptone or casamino acids stimulates the production of large numbers of conidiospores. An experiment was carried out substituting disodium ethylenediamine tetraacetic acid for protopeptone in Reese's medium.

The data, in Table XXIII show that it does stimulate both conidiospore formation and protein synthesis, although not as well as protopeptone. Thus, the effect is probably at least partly a chelation phenomenon rather than a nutritional one.

A large experiment was then set up in order to establish the best combination of ingredients for protein synthesis and cellulose utilization from ball-milled newspaper in media based on Reese's medium and Medium G. Table XXIV gives the data. In general, the best yields came from P9 and P10 in Medium G-based media supplemented with urea and yeast autolysate or casamino acids. Culture P26 produced little or no protein in the Medium G-based media, but gave good yields in nearly all of the Reese's medium variations. There were striking differences in terminal pH. Nearly all of the Reese's medium variations gave low pH values. The range was pH 2.9 to 7.0, with more than half the cultures between 2.9 and 3.6. The Medium G variations ranged from pH 3.6 to 7.8, with more than half at 7.0 to 7.8. Those flasks with high urea concentration (0.06%) gave high pH values. Because of a malfunction in a temperature regulator on one of the shaker-incubators, 22 of the 140 flasks were incubated at temperatures up to 37 C. The protein yields in these flasks were generally low. Protein yields were also low in cultures with terminal pH values above 7.4.

Shaking Rate

An experiment was carried out to determine the influence of shaking rate on protein synthesis. The three rates employed were 175, 264 and 350 RPM. The three fungus cultures P9, P10 and P26, were grown on Reese's medium, supplemented with casamino acids instead of protopeptone, and 2% ball-milled Denver Post. The data, given in Table XXV indicate little difference. The two lower rates of shaking give slightly higher protein yields than the high rate for cultures P9 and P26, and the same yield for P10, within experimental error.

Concentration of Paper and of Nitrogen Source

An experiment was then carried out to evaluate the effect of paper concentrations. (2, 4, 8 and 16 g per 100 ml of modified Reese's medium) and nitrogen source (1.4 g/l vs. 14 g/l of ammonium sulfate). The data, given in Table XXVI indicate that protein yield increases with increasing paper concentration up to 8 g/100 ml at a nitrogen concentration of 1.4 g/l of ammonium sulfate, and then drops off at 16 g/l. At 14 g/l of ammonium sulfate, protein yields are higher in most cases, particularly in the flasks containing 16 g/100 ml of paper.

A similar experiment was set up using modified medium G. The results, given in Table XXVII, were distinctly different, in that the protein yields were larger in the low nitrogen concentration (10 g/l) than in the high (50 g/l), in most cases. Yields generally increased with increasing paper concentration up to 16 g/100 ml, but were less than proportional to paper concentration, i.e. 16 g never gave 8 times the protein yield of 2 g.

Effect of Tween-80

Since Reese and McGuire[13] have reported that the presence of Tween-80 in the culture medium increases the rate of cellulase production by Trichoderma viride, the effect of this surfactant (sorbitan monooleate) was investigated by incorporating it into Reese's medium and medium G containing 2% newspaper at levels of 0.01, 0.1 and 0.5 g/l. The data, given in Table XXVIII, indicate slight stimulation of 5-day protein yield at the two lower levels in Reese's medium, but slight inhibition at the highest level. It is interesting to note that Tween-80 had no stimulatory effect on protein yield in medium G, but did exhibit a slight inhibitory effect at the highest level as it did in Reese's medium.

Since heated and ball-milled newspaper was shown (Table XXII) to give higher protein yields than unheated ball-milled newspaper, it was hoped that heating alone might improve protein yields to the point where ball-milling might be unnecessary. In order to evaluate this point, a comprehensive experiment was set up using medium G as well as medium G supplemented with urea & with urea and yeast autolysate, along with either coarsely shredded newspaper, shredded and heated newspaper, or ball-milled newspaper. First a series was harvested and analyzed after 5 days' incubation. The data, given in Table XXIX, indicate much higher protein yields from ball-milled newspaper. In view of the possibility that slower growth of the fungi on shredded paper might be responsible for the results, the entire experiment was repeated, with 3 times as many flasks; one series was analyzed after 5 days, a second after 9 days and a third after 14 days. The data, given in the same Table, do show considerably higher protein values after 14 days from the shredded and shredded and heated paper, but again the yields from the ball-milled paper are far superior, regardless of incubation time. The shredded-heated-paper did not give significantly higher protein yields than unheated-shredded-paper.

Scale-up Studies in Stirred-Jar Fermentors

It is well known in the fermentation industry that results obtained in shake flasks are not necessarily duplicated in larger stirred-jar fermentors. They do, however, serve as useful guides for such work, and many variables such as pH, nitrogen sources, buffers and nutrients can be approximately optimized in shake flask studies before proceeding to the more time-consuming stirred-jar work.

Stirred-jar studies were carried out in a New Brunswick Scientific Co. Model CMF 314 microferm floor fermentor consisting of three 14-liter fermentors in a constant temperature bath. The fermentors were equipped with automatic foam sensing and defoamant addition controls and automatic constant - pH titration equipment. Figure 1 illustrates the equipment in use. The entire fermentor, with culture media and all accessory devices and tubing included, was autoclaved for 30 minutes at 20 psig for sterilization. The volume of medium in each jar was 6.57 to 10 liters, and the temperature was controlled at 30 C. Air was sterilized by filtration through a 15 cm

by 2.0 cm column of packed sterile absorbent cotton. Samples were collected every working day for analytical and pH determinations. Table XXX summarizes the variables investigated in a total of 12 runs, and the final protein yields and cellulose consumption. The first two runs were not included as difficulties with temperature control and air filtration in these preliminary runs gave erratic results. Results are also presented graphically in Figures 2-19. Run 10 was not graphed because of solids - build-up problems.

A study of the Table and the figures leads to the following conclusions:

1. Culture P9, Myrothecium verrucaria, grows well on ball-milled newspaper, actively consumes cellulose and synthesizes cell material containing protein.

2. The optimum stirring rate for protein synthesis is 300 to 400 RPM, higher stirring rates could not be employed because of excessive splashing and foaming, leading to excessive build-up of solids on the walls of the fermentor above the normal liquid level.

3. The optimum aeration rate depends partially upon stirring rate, as oxygen transfer increases with increased stirring rate. From 3 to 6 l/min appears to be optimal at 300 to 400 RPM. At 100 RPM, 6 l/min is superior to lower air rates. Again, very high air rates must be avoided because of foaming and solids-build-up problems.

4. The pH optimum was broad, from 3.9 to 6.5.

5. The incorporation of 0.03% urea into the medium G prevented the pH from dropping below pH 4.8, and maintained it within the optimum range. Urea also increased the protein yield.

6. The incorporation of yeast autolysate in addition to urea greatly stimulated both growth rate and protein yield.

7. The maximum rate of protein synthesis was obtained in run 11, jar 1. A protein yield of 1.2 mg/ml was obtained in 4 days, at which time 54% of the cellulose originally present had been consumed. From these data, the calculated rates of cellulose utilization and protein biosynthesis are:

Cellulose consumed, g/l/day	5.4
Protein produced g/l/day	0.3
Protein, % yield as % of cellulose consumed	5.6

8. The protein yield increases with increasing paper concentration up to 8 g per 100 ml of medium, the highest level tested.

The highest final protein yields, 1.42 and 1.36 mg/l (which are probably the same, within experimental error) were obtained in stirred-jar number 1 of run 12 and 3 of run 10 respectively, both in medium G3. Jar 3 of run 10 was at the highest air rate employed, 6.0 l/min, and the highest stirring rate, 400 RPM at the start, reduced to 300 RPM after 48 hours. Thus, oxygen transfer rate, which is increased by both air rate and stirring rate, is limiting growth. Higher air rates or stirring rates could not be used because of problems encountered with foaming and solids build-up on the walls of the fermentor above the normal liquid level. The solids build-up problem in run 10 was so severe that a large proportion of the fermentor solids were clinging to the walls of the fermentor; consequently it was impossible to obtain satisfactory protein analyses on samples collected during

the run, and all of the protein analyses from day 3 through day 10 were very low - in the range of 0.2 to 0.5 mg/ml. At the termination of the experiment, the solids were scraped back into the fermentor, and thoroughly mixed, so that the final analyses would be accurate.

Run 12 clearly indicated the beneficial effect of the yeast autolysate in the medium G-3 on protein synthesis. In general high protein yield, and high rate of protein synthesis correlated well with cellulose utilization.

It is interesting to speculate whether better results could be obtained at higher substrate concentrations. Runs 8, 9 and 10 clearly indicate that the oxygen transfer rate is limiting protein synthesis at 4 g of ball-milled newspaper per 100 ml of medium. Oxygen demand increases with increasing substrate concentration, as does the viscosity of the medium. The viscosity increase will decrease the oxygen transfer rate. Thus, it is possible that further increases in the substrate concentration might not give higher protein yields or higher rates of protein synthesis. Jar 3 of run 7, where 8 g of substrate per 100 ml of medium was employed, is a case in point. Even after 24 days' incubation the protein yield was only 0.63 mg/ml, probably as a result of insufficient oxygen transfer rate.

Composition of Final Product

In order to evaluate the final product from run 10, the fermentor contents of Jar 3 were evaporated to dryness by boiling, with stirring, on an electric hot plate in a 5 l stainless steel beaker. When the total volume was reduced to 1-2 l, the material was placed in a pyrex glass dish, and evaporated to dryness in an oven at 90-100 C. The dried cake was then ball-milled into a fine powder for analysis.

Moisture content was determined by the A.O.A.C. method,[17] p.327 after equilibration of the ball-milled material with the atmosphere at ambient temperature and humidity for several hours. Ash content was determined gravimetrically by ignition in a Bunsen burner flame.

A suspension of this material, 1 g per 100 ml, was prepared and an aliquot analyzed for total protein by the "revised" Biuret method. Following centrifugation of this suspension, aliquots of supernatant were analyzed for soluble protein in a similar manner.

Ammonia nitrogen and organic nitrogen were estimated by the A.P.H.A. method, pages 240-242[18] and A.O.A.C., page 16[17] respectively. Total Kjeldahl nitrogen was calculated as the sum of the ammonia and organic nitrogen values. Nitrate nitrogen was determined colorimetrically by a brucine reaction[19]. Nitrite nitrogen was determined colorimetrically by a diazotization method[19].

Total carbohydrates were determined on a sample of the material dissolved in 67% H_2SO_4 by the anthrone reaction[5]. Soluble carbohydrates

were measured similarly on a centrifuged sample of the material. Cellulose content was estimated according to the method established in this laboratory[6]. Total lipids were estimated by the A.O.A.C. method, pages 197-198[17]. The data are summarized in Table XXXI, along with similar data on corn and wheat[20], both of which are excellent feed materials for cattle, although slightly low in protein to make a complete food for human consumption. It can be seen that our material is higher in ash and cellulose, and somewhat lower in protein. It is likely that these factors would not limit its use as a cattle feed, however.

Discussion

The screening phase of this project reexamined the occurence of rapidly-growing cellulolytic microorganisms in nature. The methods used deliberately selected only those organisms which were capable of a high rate of protein synthesis in submerged culture. As might have been expected, the organisms isolated were from genera long known to be actively cellulolytic, Myrothecium verrucaria, Trichoderma viride, Aspergillus fumigatus and Cellulomonas. As pointed out by Gascoigne and Gascoigne[1], the 19th century biochemist, Hoppe-Seyler, had studied cellulase production by fungi, and the 19th century mycologist, DeBary described several cellulolytic fungi.

In our experiments, Myrothecium verrucaria produced the highest yields of protein, and hence our optimization work was carried out with this organism. This is in agreement with many reports in the literature[21] emphasizing the very active cellulolytic properties of this fungus.

By comparison with cellulose, lignin is very slowly attacked by microorganisms[24], and it is probable that little or no lignin was consumed in any of our experiments, although this cannot be stated with certainty, since direct analyses for lignin were not carried out.

Our data are not sufficient to make a material balance, but some hypothetical calculations of this type may be given for jar 1 run 12, which gave the highest protein yield.

Paper originally present, g/l	40
Protein, g/l (from biuret analysis)	1.42
Protein, g/l (from organic N x 6.25)	3.3
Cellulose original present, g/l	20.4
Cellulose consumed, g/l	12.7

Assume that 50% of the 12.7 g of cellulose substrate consumed was oxidized for energy, and 50% was assimilated and converted to cell material which contains 30% protein. (These values were taken from Gray[11] as being fairly typical for fungi imperfecti grown on glucose.) Then the amount of cellulose converted to cell material would be 6.35 g/l and the protein would be 1.91 g/l. Gray determined protein as organic N x 6.25. Thus, it can be seen that our corresponding protein value, 3.3 g/l, is considerably higher than the example given by Gray. The true protein value of our product (as well as Gray's) is probably closer to the biuret value of 1.42 g/l, since N x 6.25 includes in addition to true protein all other nitrogen compounds found in the cells such as amino acids, phospholipids, purines and pyrimidines DNA and RNA. In any event, returning to our

product calculations beginning with 40 g/l of newspaper, it should be possible to produce 33.7 g/l of final product containing 3.3 g/l of "protein" calculated as N x 6.25.

The work accomplished was nearly sufficient to permit the design and construction of a pilot plant, although a few more stirred-jar runs using higher concentrations of ball-milled paper and perhaps higher concentrations of ammonium phosphate and urea may be desirable. Although Ciegler and Lillhoj[25] do not list Myrotheciúm verrúcaria among the mycotoxin-producing fungi, some preliminary feeding studies with mice and rats on our final products would be highly desirable before proceeding with pilot plant construction in order to obtain some evaluation to toxicity and dietary value.

Table I

The Utilization of Cellulose by Enrichment Cultures

Serial No.	Culture No.	Medium a=Newspaper b=Purified cellulose c=Newsprint (1g/100 ml)	Medium Enrichment M=Mineral salts E=Organic nutrients HC=Hydrocarbon enrichment	Cellulase Assay	Final Cell Count per ml	Protein mg/ml (Biuret)	% of Cellulose Utilized*
1	353x114-30	Pl a	M	-	8.9×10^7	1.38	31
2	353x114-30	Pl c	M	-	4.3×10^7	0.29	4
3	353x114-30	SM a	E	-	3.2×10^9	0.35	4
4	353x114-30	Pl a	M	-	7.3×10^7	0.62	X
5	353x99-B	SM a	E	+	3×10^6	0.53	54
6	353x99-B	SM b	E	+	3×10^6	0.75	59
7	353x99-B	SM c	E	+	3×10^6	0.58	56
8	353x113-8	SM b	E	-	2.2×10^9	0.25	3
9	353x113-10	SM a	E	+	1.1×10^8	0.53	52
10	353x113-10	SM c	E	+	4×10^6	0.66	59
11	353x113-11	SM a	E	+	1.0×10^9	0.51	45
12	353x113-11	SM b	E	-	5.5×10^9	0.55	27
13	353x113-11	SM c	E	-	7.7×10^9	0.44	19
14	353x113-15	SM a	E	-	2.4×10^9	0.20	6
15	353x113-15	SM b	E	-	4.6×10^9	0.28	5
16	353x113-15	SM c	E	-	5.7×10^9	0.34	25
17	353x113-19	SM b	E	-	1.9×10^9	0.22	9
18	353x119-19	SM c	E	-	1.2×10^9	0.31	4
19	353x113-24	SM a	E	-	1.6×10^9	0.39	34
20	353x113-24	SM b	E	+	4.1×10^9	0.37	5
21	353x113-24	SM c	E	+	5.9×10^9	0.36	17
22	353x113-25	SM a	E	+	4.5×10^9	0.56	24
23	353x113-25	SM b	E	+	8.1×10^9	0.36	14
24	353x113-25	SM c	E	-	6.8×10^9	0.55	28
25	353x113-8	Reese a	E	-	5.4×10^9	0.60	X
26	353x113-8	Reese b	E	-	1.9×10^9	0.28	36
27	353x113-8	Reese c	E	+	1.3×10^9	0.28	23
28	353x113-10	Reese a	E	+	1.7×10^8	0.75	X
29	353x113-10	Reese b	E	+	9×10^6	0.85	81

Table I (cont.)

Serial No.	Culture No.	Medium a=Newspaper b=Purified cellulose c=Newsprint	Medium Enrichment M=Mineral salts E=Organic nutrients	Cellulase Assay	Final Cell Count per ml	Protein mg/ml (Biuret)	% of Cellulose Utilised*
30	353x113-10	Reese c	E	+	4×10^6	0.59	65
31	353x113-11	Reese a	E	-	4.6×10^9	0.38	X
32	353x113-11	Reese b	E	-	TNTC	0.98	14
33	353x113-15	BH a	M	-	1.4×10^9	0.23	X
34	353x113-18	BH c	M	-	2.7×10^8	0.36	29
35	353x113-24	BH a	M	-	1.1×10^{10}	0.44	20
36	353x113-24	BH b	M	-	8.1×10^9	0.39	20
37	353x113-25	BH a	M	-	3.2×10^9	0.40	34
38	353x113-25	BH b	M	-	1.0×10^{10}	0.48	15
39	353x113-30	SM a	E	+	1.1×10^9	0.47	20
40	353x113-30	SM b	E	-	1.1×10^9	0.23	3
41	353x113-30	SM c	E	-	1.6×10^9	0.21	7

X = Analysis not done or not included because of known error.

* As % of original cellulose in sterile control from 1 g of cellulosic substrate in 100 ml of culture medium.

SM = Srinivasan's medium.

BH = Bushnell-Haas medium.

Table II

The Utilization of Cellulose by Pure Cultures

Serial No.	Culture No.	Medium a=Newsprint b=Purified cellulose c=Newspaper (1g/100 ml)	Medium Enrichment M-Mineral salts E-Organic nutrients	Cellulase Assay	Final Cell Count per ml	Protein mg/ml (Biuret)	% of Cellulose Utilized
1	P 9	SM a	E	+	TNTC	0.52	47
2	P 9	SM c	E	+	8.1×10^7	0.26	30
3	P 10	SM c	E	+	7.6×10^7	0.28	8
4	353x85-1	SM a	E	+	4.0×10^6	0.22	X
5	353x85-1	SM b	E	+	6.0×10^6	0.49	X
6	353x85-1	SM c	E	+	2.0×10^6	0.40	X
7	353x99-B	Reese a	E	+	9.0×10^6	0.81*	54
8	353x99-B	Reese b	E	+	3.0×10^6	0.84+	78
9	353x99-B	Reese c	E	+	1.4×10^6	0.32	34
10	C 264	SM a	E	X	Heavy fungus growth	0.44	X

* Biuret value not corrected for blank

\+ Biuret value too high because of interfering turbidity

X Analysis not done or not included because of known error

Table III

Characteristics of Cellulolytic Cultures from Tables I and II

	Culture No.	Pure (P) or Enrichment (E)	Serial No.	Source	Description
1	353 x 113-30	E	1,2,3,4,39,40,41	Soil S1	Mixed bacteria
2	353 x 99-B*	E	5,6,7	Aerobic sewage sludge	Fungus
3	353 x 113-8	E	8,25,26,27	Soil 55	Mixed bacteria
4	353 x 113-10	E	9,10,28,29,30	Soil 58	" "
5	353 x 113-11	E	11,12,13,31,32	Aerobic sewage sludge	" "
6	353 x 113-15	E	14,15,16,33	Soil 49	" "
7	353 x 113-18	E	34	Soil 52	" "
8	353 x 113-19	E	17,18	Soil 53	" "
9	353 x 113-24	E	19,20,21,35,36	Aerobic sewage sludge	" "
10	353 x 113-25	E	22,23,24,37,38	Anaerobic sewage sludge	" "
1	P 9	P	1,2	Soil No. 53	Fungus, identified as Myrothecium verrucaria
2	P 10	P	3	Soil No. 53	Same
3	353 x 85-1	P	4,5,6	Compost soil No. 59	Fungus, identified as Aspergillus fumigatus
4	353 x 99-B*	P	7,8,9	Aerobic sewage sludge	Fungus, identified as Trichoderma lignorum
5	C 264+	P	10	HC ox.+	Fungus, identified as Aspergillus fumigatus

+ Isolated on the hydrocarbon, n-hexadecane

* After one series of fermentations, this culture was established to be pure.

Table IV

The Optimization of Conditions for Growth Protein Synthesis and Cellulose Utilization by Pure Cultures of Fungi on 1% Newsprint

Serial No.	Culture	Med.+	Temp.	Time of Inc., Days	Micro-scopic Exam.*	pH	Protein mg/ml	% Cellulose Decomposed
1	P-9	TV	30	2	a3+	4.8	0.41	59
2		TV	37	2	a3+	4.8	0.31	70
3		TV	45	2	No growth	5.3	0.04	-
4		SM	30	2	a3+	3.6	0.22	45
5		SM	37	2	a3+	4.7	0.15	38
6		SM	45	2	No growth	5.7	0.03	-
7		TV	30	5	a3+	4.4	0.48	75
8		TV	37	5	a3+	5.5	0.37	75
9		TV	45	5	No growth	5.9	0.06	-
10		SM	30	5	a3+	3.0	0.45	60
11		SM	37	5	a2+	3.6	0.34	65
12		SM	45	5	No growth	5.7	0.08	-
13		TV	30	9	a3+	3.7	0.41	77
14		TV	37	9	a3+	5.4	0.31	84
15		TV	45	9	No growth	5.3	0.12	-
16		SM	30	9	a3+	3.0	0.48	54
17		SM	37	9	a3+	3.9	0.28	49
18		SM	45	9	No growth	5.7	0.08	-
19	P-10	TV	30	2	a3+	5.3	0.39	74
20		TV	37	2	a3+	5.3	0.27	69
21		TV	45	2	No growth	5.6	0.03	-
22		SM	30	2	a3+	3.3	0.33	44
23		SM	37	2	a3+	4.1	0.35	50
24		SM	45	2	No growth	5.7	0.05	-
25		TV	30	5	a3+	4.2	0.49	75
26		TV	37	5	a3+	5.3	0.38	81
27		TV	45	5	No growth	5.7	0.10	-
28		SM	30	5	a3+	3.1	0.44	48
29		SM	37	5	a3+	3.7	0.34	55
30		SM	45	5	No growth	5.8	0.07	-

Table IV (cont.)

Serial No.	Culture	Med.+	Temp.	Time of Inc., Days	Micro-scopic Exam.*	pH	Protein mg/ml	% Cellulose Decomposed
31	P-10	TV	30	9	a3+	4.0	0.35	76
32		TV	37	9	a3+	5.3	0.29	85
33		TV	45	9	b2+	5.6	0.19	81
34		SM	30	9	a3+	3.2	0.43	54
35		SM	37	9	3+	4.2	0.30	61
36		SM	45	9	No growth	5.7	0.08	-
37	353x85-1	TV	30	2	b3+	6.3	0.04	-
38		TV	37	2	b3+	6.5	0.11	-
39		TV	45	2	b3+	5.4	0.27	52
40		SM	30	2	b3+	5.8	0.06	-
41		SM	37	2	b3+	5.1	0.25	16
42		SM	45	2	b3+	4.9	0.25	49
43		TV	30	5	b3+	5.2	0.26	59
44		TV	37	5	b3+	5.5	0.40	70
45		TV	45	5	b3+	5.0	0.15	-
46		SM	30	5	b3+	4.8	0.19	-
47		SM	37	5	b3+	5.1	0.34	66
48		SM	45	5	b2+	4.6	0.29	72
49		TV	30	9	b3+	5.4	0.36	78
50		TV	37	9	b2+	5.4	0.31	80
51		TV	45	9	b3+	6.2	0.13	81
52		SM	30	9	b2+	4.9	0.40	77
53		SM	37	9	b2+	5.2	0.36	80
54		SM	45	9	b2+	5.8	0.28	81
55	353x99B	TV	30	2	b3+	3.9	0.31	62
56		TV	37	2	No growth	6.1	0.01	-
57		TV	45	2	No growth	5.7	0.03	-
58		SM	30	2	b3+	2.9	0.48	58
59		SM	37	2	b2+	6.0	0.04	-
60		SM	45	2	No growth	5.6	0.05	-
61		TV	30	5	b3+	5.5	0.35	75
62		TV	37	5	b2+	6.1	0.12	-
63		TV	45	5	b2+	5.3	0.07	-
64		SM	30	5	b3+	3.1	0.45	71
65		SM	37	5	b3+	4.5	0.19	-

Table IV (cont.)

Serial No.	Culture	Med.+	Temp.	Time of Inc., Days	Micro-scopic Exam.*	pH	Protein mg/ml	% Cellulose Decomposed
66	353x99B	SM	45	5	b2+	5.5	0.05	-
67		TV	30	9	b3+	5.6	0.35	83
68		TV	37	9	b3+	5.9	0.05	-
69		TV	45	9	No growth	5.6	0.07	-
70		SM	30	9	b2+	3.2	0.45	68
71		SM	37	9	b3+	5.5	0.16	-
72		SM	45	9	b2+	5.1	0.29	76
73	C-264	TV	30	2	b3+	5.3	0.23	38
74		TV	37	2	b3+	5.3	0.38	63
75		TV	45	2	b3+	5.8	0.32	65
76		SM	30	2	b3+	4.0	0.27	49
77		SM	37	2	b3+	4.6	0.23	39
78		SM	45	2	b3+	5.1	0.21	61
79		TV	30	5	b3+	5.3	0.28	73
80		TV	37	5	b3+	5.4	0.31	77
81		TV	45	5	b3+	6.2	0.20	-
82		SM	30	5	b3+	4.4	0.28	64
83		SM	37	5	b3+	4.4	0.31	61
84		SM	45	5	b2+	5.8	0.26	72
85		TV	30	9	b3+	5.4	0.26	84
86		TV	37	9	b3+	6.0	0.38	82
87		TV	45	9	b+	6.5	0.13	-
88		SM	30	9	b+	5.0	0.31	77
89		SM	37	9	b2+	5.5	0.37	79
90		SM	45	9	b+	5.9	0.23	82
91	Sterile Control	TV	30	2	No growth	5.5	-	-
92		TV	37	2		5.4	-	-
93		TV	45	2		5.6	-	-
94		SM	30	2		5.5	-	-
95		SM	37	2		5.6	-	-
96		SM	45	2		5.6	-	-
97		TV	30	5		5.6	-	-
98		TV	37	5		5.7	-	-
99		TV	45	5		5.7	-	-
100		SM	30	5		5.5	-	-
101		SM	37	5		5.5	-	-

Table IV (cont.)

Serial No.	Culture	Med.+	Temp.	Time of Inc., Days	Micro-scopic Exam.*	pH	Protein mg/ml	% Cellulose Decomposed
102	Sterile Control	SM	45	5	No growth	5.6	-	-
103	↓	TV	30	9		5.4	-	-
104		TV	37	9		5.4	-	-
105		TV	45	9		5.4	-	-
106		SM	30	9		4.3	-	-
107		SM	37	9		4.9	-	-
108		SM	45	9		5.3	-	-

* Microscopic examination:
  a = mostly oval yeast-like cells
  b = Filamentous mycelium

+ Media:
  TV = Reese's medium
  SM = Srinivasan's medium (See Appendix)

Each flask was inoculated with 1 ml of a 48-hour shake flask culture, grown as follows:

P-9 and P-10	SM medium + 1% newsprint, 30 C
353x85-1	TV medium + 1% newsprint, 45 C
353x99-B	TV medium + 1% newsprint, 30 C
C-264	SM medium + 1% newsprint, 37 C

Table V

The Influence of Paper Concentration on the Growth of Fungi

Serial No.	Culture	Med.+	Microscopic Examination* 2 days	5 days	pH
1	P-9	A	No growth	Fair growth	2.6
2	P-9	B	Good growth	Good growth	2.6
3	P-9	C	No growth	Good growth	2.6
4	P-10	A	No growth	Fair growth	2.7
5	P-10	B	Good growth	Good growth	2.5
6	P-10	C	No growth	Fair growth	2.6
7	353x99B	A	No growth	Fair growth	3.4
8	353x99B	B	No growth	Fair growth	2.3
9	353x99B	C	No growth	No growth	3.7
10	Controls	A	No growth	No growth	3.9
11	Controls	B	No growth	No growth	4.7
12	Controls	C	No growth	No growth	3.7
13	P9	D	Good growth	Good growth	6.5
14	P-10	D	Good growth	Good growth	6.3
15	353x99B	D	No growth	No growth	6.3
16	P-9	E	No growth	Fair growth	5.9
17	P-10	E	No growth	Good growth	5.6
18	353x99B	E	No growth	Good growth	6.6
19	Control	D	No growth	No growth	4.9
20	Control	E	No growth	No growth	4.6

* All cultures were examined at 2 days' and 5 days' incubation.
Incubated at 30 C
Inoculum: Growth from a nutrient agar slant.

+ Media: A 30 g Newspaper, ball-milled (17% by weight paper)
All media B 30 g Purified cellulose (17% by weight cellulose)
were made C 30 g Newsprint, ball-milled (17% by weight paper)
from 150ml D 1.5 g Newsprint, blended (0.99% by weight paper)
of Reese's E 2.0 g Newsprint, blended (1.3% by weight paper)
medium,with
the following
additions

Table VI

Simplification of Culture Media for Paper Digestion and Protein Synthesis

Serial No.	Culture	Med.+	Inc. Time, days	Microscopic Exam.	pH	Protein mg/ml
1	P-9	F	2	Fair growth	3.1	0.06
2		G	2	Good "	4.9	0.13
3		SM	2	Good "	2.8	0.37
4		F	5	Fair "	2.8	0.52
5		G	5	Fair "	3.3	0.75
6		SM	5	Good "	2.5	0.55
7		F	9	Good "	2.9	0.44
8		G	9	Good "	3.9	0.83
9		SM	9	Good "	2.5	0.49
10	P-10	F	2	Fair "	3.1	0.10
11		G	2	Fair "	5.4	0.10
12		SM	2	Fair "	3.5	0.18
13		F	5	Fair "	2.8	0.37
14		G	5	Good "	3.2	0.69
15		SM	5	Good "	2.4	0.57
16		F	9	Fair "	2.7	0.52
17		G	9	Good "	3.4	0.90
18		SM	9	Good "	2.6	0.42
19	353x99B	F	2	Good "	2.5	0.39
20		G	2	Good "	2.8	0.68
21		SM	2	Good "	2.2	0.65
22		F	5	Good "	2.5	0.35
23		G	5	Fair "	2.9	0.60
24		SM	5	Good "	2.6	0.28
25		F	9	Good "	2.7	0.41
26		G	9	Good "	3.3	1.08
27		SM	9	Good "	2.5	0.52
28	Control	F	2	None	4.1	
29		G	2		5.3	
30		SM	2		5.7	
31		F	5		4.2	
32		G	5		5.4	
33		SM	5		5.7	

Table VI (cont)

Serial No.	Culture	Med.+	Inc. Time, days	Microscopic Exam.	pH	Protein mg/ml
34	Control	F	9	None	4.3	-
35	↓	G	9	↓	5.3	-
36		SM	9		5.6	-

+ Media: F 100 ml tap water + 1 g $(NH_4)_2SO_4$ + 2 g of balled-milled newspaper (Wall St. Journal)

G 100 ml tap water + 1 g $(NH_4)_2HPO_4$ + 2 g of ball-milled newspaper (WSJ)

SM 100 ml of Srinivasan medium + 2 g of ball-milled newspaper (WSJ)

Inoculum: 5 day old shake flask cultures from Table V. (Reese's medium plus 17% newsprint)

Flask 3 - Inoculum for cultures 1-9

Flask 6 - Inoculum for cultures 10-18

Flask 7 - Inoculum for cultures 19-27

Table VII

Growth and Protein Synthesis by Cellulolytic Fungi Growing on Medium H

Flask No.	Inoculum	Temp. °C	Time, Days	Terminal pH	Protein mg/ml
1	P9	30	2	3.5	0.11
2	"	"	5	3.4	0.15
3	"	"	9	3.6	0.30
4	P10	"	2	3.5	0.13
5	"	"	5	3.3	0.20
6	"	"	9	4.1	0.33
7	P26	"	2	3.4	0.15
8	"	"	5	3.8	0.11
9	"	"	9	3.7	0.07
10	C264	37	2	2.9	0.43
11	"	"	5	3.1	0.28
12	"	"	9	3.7	0.31
13	353x85-1	45	2	3.4	0.18
14	"	"	5	3.1	0.19
15	"	"	9	3.1	0.30
16	Control	30	2	4.8	-
17	"	37	2	4.8	-
18	"	45	2	4.8	-
19	"	30	5	4.9	-
20	"	37	5	4.8	-
21	"	45	5	4.8	-
22	"	30	9	5.2	-
23	"	37	9	5.2	-
24	"	45	9	5.2	-

Inoculum: 1 ml of a 9 day shake flask culture from the experiment of Table VI, growing in Medium G with 2% ball-milled Wall Street Journal.

Table VIII

Characteristics of Cellulolytic Bacteria

Tube No.	Inoculum	Filter Paper Strip Test		CMC hydrolysis*	Agar Stroke	Nut. broth	Methyl Red	Vogues Pros sauer	Indole
		5 das	14 das	5 das					
1	X125-1	+	3+	3+	Filiform	Mod.turb.	Neg.	Neg.	Neg.
2	X125-2	+	3+	3+	"	"	"	"	"
3	X125-3	+	3+	3+	"	"	"	"	"
4	X125-4	+	3+	3+	"	"	"	"	"
5	Cellulomas sp.	+	3+	3+	"	"	"	"	"
6	Control	0	0	0	"	"	"	"	"

*CMC medium was prepared by adding 4.9% of sodium carboxymethyl cellulose (Hercules type 7MF). A 3+ reaction indicates complete liquefaction of the entire tube of viscous medium. 3+ on the filter-paper strip test means dissolution of the paper into a small amount of fibrous material.

All were small Gram variable, non-motile rods which did not ferment glucose.

Table IX

Growth and Protein Synthesis by Cellulomonas and Related Bacterial Cultures Growing on Ball-Milled Paper or Purified Cellulose in Srinivasan's Medium Incubated at 37 C.

Flask No.	Inoculum	Inc. time days	Substrate +	Term pH 37 C	Protein mg/ml 30 C	Protein mg/ml 37 C	Microscopic Exam.
1	353x125-1	5	A	4.7		.03	
2	353x125-2		A	4.9		.03	
3	353x125-3		A	4.9		.05	
4	353x125-4		A	4.9		.03	
5	Cellulomonas		A	5.0		.03	
6	353x125-1		B	4.7		.07	
7	353x125-2		B	4.8		.07	
8	353x125-3		B	4.8		.07	
9	353x125-4		B	4.8		.07	
10	Cellulomonas		B	5.0		.07	
11	353x125-1		C	6.0		.02	Nothing
12	353x125-2		C	6.0		-.01	
13	353x125-3		C	6.0		-.01	
14	353x125-4		C	6.0		-.01	
15	Cellulomonas		C	6.0		.01	
16	Control		A	6.0		-	
17	"		B	6.0		-	
18	"		C	6.0		-	

+ Substrate:

A - Srinivasan's med. + 2% ball-milled newspaper (Wall St. Journal)

B - Srinivasan's med. + 2% purified cellulose

C - Srinivasan's medium + 2% newsprint

Table X

Growth and Protein Synthesis by Trichoderma Viride Grown on Ball-Milled Newsprint in Reese's and Srinivasan's Media at 30 and 37 C

Time, Days	Medium*	Inc.	Term pH 30 C	Term pH 37 C	Protein mg/ml 30 C	Protein mg/ml 37 C	Cellulose Utilized % 30 C	Cellulose Utilized % 37 C
-1	b	2	6.5	4.6	-0.03	-0.04		
-2	a	2	6.5	6.7	0.05	0		
-3	b	5	6.0	6.2	0.43	0.17		87%
-4	a	5	2.7	6.7	0.54	0.03		
-5	b	11	4.9	6.5	0.46	0.29		90%
-6	a	9	2.8	6.6	0.51	0		
-7	b	2	5.0	5.2	-	-		
-8	a	2	5.9	5.7	-	-		
-9	b	5	4.9	5.2	-	-		
-10	a	5	5.9	5.7	-	-		
-11	b	11	6.8	5.1	-	-		
-12	a	9	5.9	5.6	-	-		

Media a Srinivasan's medium + 1% newsprint

b Reese's medium + 1% newsprint

Table XI

Chemical Treatment of Pulped Newspaper

(Wall Street Journal)

Chemical Treatment		Wt. of paper before treatment, g	Wt. of paper recovered after treatment, g
H_2O	Hot	9.83	9.60
H_2O	Cold	9.71	9.56
NaOH	Hot	9.66	7.53
NaOH	Cold	9.78	9.21
H_3PO_4	Hot	9.86	8.38
H_3PO_4	Cold	9.73	9.46
H_2SO_4	Hot	9.92	7.61
H_2SO_4	Cold	9.85	9.57
$NaHSO_3$	Hot	9.73	10.06
$NaHSO_3$	Cold	9.67	10.00

Table XII

Growth and Protein Synthesis on Chemically Treated Papers in Medium H in Five Days

Flask No.	Culture No.	Paper Treatment	Temp. °C	Term. pH	Protein mg/ml
1	P9	H_2O Hot	30	3.9	0.09
2	"	H_2O Cold	"	4.1	0.03
3	"	NaOH Hot	"	4.2	0.05
4	"	NaOH Cold	"	4.1	0.06
5	"	H_3PO_4 Hot	"	4.1	0.09
6	"	H_3PO_4 Cold	"	3.8	0.08
7	"	H_2SO_4 Hot	"	4.5	0.03
8	"	H_2SO_4 Cold	"	3.9	0.11
9	"	$NaHSO_3$ Hot	"	3.8	0.08
10	"	$NaHSO_3$ Cold	"	3.8	0.08
11	P10	H_2O Hot	"	3.9	0.11
12	"	H_2O Cold	"	4.0	0.05
13	"	NaOH Hot	"	4.6	0.03
14	"	NaOH Cold	"	4.3	0.07
15	"	H_3PO_4 Hot	"	4.0	0.09
16	"	H_3PO_4 Cold	"	4.0	0.08
17	"	H_2SO_4 Hot	"	4.5	0.06
18	"	H_2SO_4 Cold	"	4.0	0.11
19	"	$NaHSO_3$ Hot	"	3.9	0.07
20	"	$NaHSO_3$ Cold	"	3.8	0.09
21	353x85-1	H_2O Hot	45	3.8	0.13
22	"	H_2O Cold	"	4.0	0.07
23	"	NaOH Hot	45	4.6	0.08
24	"	NaOH Cold	"	4.2	0.08
25	"	H_3PO_4 Hot	"	4.0	0.13
26	"	H_3PO_4 Cold	"	3.9	0.09
27	"	H_2SO_4 Hot	"	4.6	0.02
28	"	H_2SO_4 Cold	"	4.0	0.10
29	"	$NaHSO_3$ Hot	"	3.9	0.07
30	"	$NaHSO_3$ Cold	"	3.9	0.09
31	P26	H_2O Hot	30	4.5	0.03
32	"	H_2O Cold	"	5.0	0
33	"	NaOH Hot	"	5.1	0.02

Table XII (cont.)

Growth and Protein Synthesis on Chemically Treated Papers in Medium H in Five Days

Flask No.	Culture No.	Paper Treatment	Temp. °C	Term. pH	Protein mg/ml
34	P26	NaOH Cold	30	4.9	0.03
35	"	H_3PO_4 Hot	"	4.4	0.04
36	"	H_3PO_4 Cold	"	4.5	0.03
37	"	H_2SO_4 Hot	"	5.2	0.02
38	"	H_2SO_4 Cold	"	4.7	0.06
39	"	$NaHSO_3$ Hot	"	4.2	lost
40	"	$NaHSO_3$ Cold	"	4.4	0.02
41	C-264	H_2O Hot	37	4.2	0
42	"	H_2O Cold	"	4.0	0
43	"	NaOH Hot	"	4.2	0
44	"	NaOH Cold	"	4.9	0
45	"	H_3PO_4 Hot	"	3.9	0
46	"	H_3PO_4 Cold	"	3.8	0
47	"	H_2SO_4 Hot	"	4.4	0
48	"	H_2SO_4 Cold	"	4.0	0
49	"	$NaHSO_3$ Hot	"	3.9	0
50	"	$NaHSO_3$ Cold	"	3.8	0
51	Control	H_2O Hot	30	5.2	-
52	"	H_2O Cold	"	5.3	-
53	"	NaOH Hot	"	5.4	-
54	"	NaOH Cold	"	5.2	-
55	"	H_3PO_4 Hot	"	5.1	-
56	"	H_3PO_4 Cold	"	5.2	-
57	"	H_2SO_4 Hot	"	5.2	-
58	"	H_2SO_4 Cold	"	5.2	-
59	"	$NaHSO_3$ Hot	"	5.1	-
60	"	$NaHSO_3$ Cold	"	5.1	-
61	*Trichoderma viride*	H_2O Hot	"	5.2	-0.02
62	"	H_2O Cold	"	5.1	-0.06
63	"	NaOH Hot	"	5.1	0.02
64	"	NaOH Cold	"	5.2	0.02
65	"	H_3PO_4 Hot	"	4.8	0.05
66	"	H_3PO_4 Cold	"	5.1	0.01

Table XII (cont.)

Growth and Protein Synthesis on Chemically Treated Papers in Medium H in Five Days

Flask No.	Culture No.	Paper Treatment	Temp. °C	Term. pH	Protein mg/ml
67	Trichoderma viride	H_2SO_4 Hot	30	5.0	-0.03
68	"	H_2SO_4 Cold	"	5.1	-0.01
69	"	$NaHSO_3$ Hot	"	4.9	-0.02
70	"	$NaHSO_3$ Cold	"	4.2	-0.01
71	P9	Ball-milled	"	3.7	0.14
72	P10	"	"	3.7	0.15
73	353x85-1	"	"	4.2	0.10
74	P26	"	"	5.1	-0.01
75	C-264	"	"	4.2	0.12
76	Trichoderma viride	"	"	3.6	0.11
77	Control	"	"	5.2	-

Inoculum: 5-day culture in Medium H with 2% ball-milled newspaper from experiment described in Table XI, except for Trichoderma viride which was a 5-day culture in Srinivasan's medium with 1% ball-milled newsprint.

Harvest: 5-day cultures in Medium H plus 1% ball-milled Wall Street Journal.

Table XIII

The Influence of the Growth Medium for the Inoculum (Reese's Medium or Medium G) on Protein Yields from Cultures Grown on Media G and H

Flask No.	Inoculum Med.	Culture	Harvest Flask Med.	Term. pH	Protein, mg/ml
1	G	P9	G	5.4	0.13
2	G	P9	H	3.4	0.57
3	R	P9	G	3.2	0.59
4	R	P9	H	3.4	0.59
5	G	P10	G	5.4	0.08
6	G	P10	H	3.8	0.16
7	R	P10	G	3.3	0.67
8	R	P10	H	3.4	0.63
9	G	P26	G	3.4	0.23
10	G	P26	H	3.4	0.29
11	R	P26	G	3.2	0.35
12	R	P26	H	3.6	0.17
13	Control	None	G	5.5	-
14	Control	None	H	5.1	-

Inoculum: 2-day cultures in medium G or Reese's medium with 2% ball-milled Wall Street Journal.
The inoculum cultures in Reese's medium showed much heavier growth under the microscope than those in Medium G.

Harvest Flasks: 5-day cultures in medium G or medium H plus 2% ball-milled Wall Street Journal.

Table XIV

The Synthesis of Protein and Cellulose by Fungus Cultures Grown on Glucose in Reese's Medium

Flask No.	Culture	Temp.	Term. pH	Protein, mg/ml	Cellulose, mg/ml
1	P9	30	2.4	0.93	0
2	P10	30	2.4	1.13	0
3	P26	30	2.4	0.75	2.7
4	C-264	45	2.3	0.42	1.4
5	Control	30	5.1	-	0

Inoculum: 2 day old culture in Medium R with 10% glucose

Harvest flask Medium: Medium R with 10% glucose, 5 days' inc.

Table XV

Protein Yields from Newspaper Chopped Dry in a Waring Blendor Compared to Ball-Milled Newspaper in Medium H

Flask No.	Culture	Temp.	Term. pH	Protein, mg/ml
1	P9	30	3.4	0.14
2	P9	30	3.4	0.14
3	C264	45	3.4	0.15
4	C264	45	3.4	0.17
5	P9	30	3.3	0.36
6	C264	45	3.5	0.25
7	Control (none)	30	4.8	-

Inoculum: 2-day culture in Medium R with 2% ball-milled newspaper (Wall Street Journal)

Harvest-Flask Media: Medium H with 2% ball-milled newspaper (Wall Street Journal) in flasks 5 and 6. Medium H with 2% newspaper chopped in Waring blendor in flasks 1-4.

Table XVI

The Effect of pH Calcium Carbonate Buffering and Trace Elements on Cellulose Utilization by Fungi in Medium G

Flask No.	Culture	Temp.	Term. pH	Trace Elements	pH Control	% Cellulose Consumed
1	P9	30	3.3	Yes	No	5
2	P10	30	3.3	"	"	24
3	P26	30	3.1	"	"	35
4	353x85-1	37	3.9	"	"	15
5	C264	37	3.4	"	"	37
6	P9	30	3.1	No	"	44
7	P10	30	3.2	"	"	35
8	P26	30	2.9	"	"	48
9	353x85-1	37	3.7	"	"	35
10	C264	37	3.8	"	"	31
11	P9	30	5.0	Yes	$CaCO_3$	44
12	P10	30	5.0	"	"	44
13	P26	30	5.2	"	"	58
14	353x85-1	37	5.2	"	"	41
15	C264	37	5.3	"	"	58
16	P9	30	5.0	No	"	60
17	P10	30	4.9	"	"	58
18	P26	30	5.1	"	"	30
19	353x85-1	37	5.3	"	"	58
20	C264	37	5.4	"	"	62
21	P9	30	5.6	Yes	Titr.	73
22	P10	30	5.5	"	"	62
23	P26	30	5.6	"	"	42
24	353x85-1	37	5.8	"	"	72
25	C264	37	5.9	"	"	67
26	P9	30	5.8	No	"	74
27	P10	30	5.8	"	"	74
28	P26	30	5.6	"	"	66
29	353x85-1	37	5.7	"	"	67
30	C264	37	5.8	"	"	63
31	Control(none)	37	4.8	Yes	No	0
32	" "	37	4.9	No	No	0

Table XVI (cont)

The Effect of pH Calcium Carbonate Buffering and Trace Elements on Cellulose Utilization by Fungi in Medium G

Flask No.	Culture	Temp.	Term. pH	Trace Elements	pH Control	% Cellulose Consumed
33	Control (none)	37	5.2	Yes	$CaCO_3$	0
34	" "	37	5.3	No	$CaCO_3$	0

Inoculum: 3-day culture in Medium R with 2% ball-milled newspaper (Wall Street Journal)

Harvest flasks: Medium G with 0.005% bromcresol green indicator and 2% ball-milled newspaper. (Wall Street Journal). Harvested at 7 days.

The pH value was maintained in the range 5-6 on flasks 21-30 by daily titration with sterile 0.1 N NaOH. (labelled "titr" in table) In flasks 11-20 pH control was achieved by incorporating 1% $CaCO_3$ in the medium.

Table XVII

The Effect of Trace Elements, Proteose Peptone and Urea on Protein Yield and Cellulose Utilization by Fungi Growing on Newspaper in Medium G

Flask No.	Inoculum Med.	Culture	Temp.	Nutrient Supplement	Term. pH	Protein mg/ml	% Cellulose Utilized
1	R	P9	30	None	3.4	0.30	19
2	"	P10	30	"	3.4	0.30	25
3	"	P26	30	"	4.2	0.06	
4	"	353x85-1	37	"	3.5	0.22	22
5	"	C264	37	"	3.7	0.26	25
6	G	P9	30	"	4.5	0.06	
7	"	P10	30	"	4.8	0.02	
8	"	P26	30	"	3.6	0.14	
9	"	353x85-1	37	"	3.6	0.18	
10	"	C264	37	"	3.5	0.26	
11	G + trace els.	P9	30	"	4.8	0.02	
12	"	P10	30	"	4.2	0.02	
13	"	P26	30	"	4.0	0.06	
14	"	353x85-1	37	"	3.6	0.22	
15	"	C264	37	"	3.5	0.26	25
16	G + trace els. + Proteose peptone	P9	30	"	3.6	0.18	
17	"	P10	30	"	3.6	0.26	19
18	"	P26	30	"	4.2	0.06	
19	"	353x85-1	37	"	3.6	0.14	
20	"	C264	37	"	3.7	0.26	19
21	R	P9	30	Trace els.	3.5	0.39	24
22	"	P10	30	" "	3.6	0.39	24
23	"	P26	30	" "	5.0	-0.03	
24	"	353x85-1	37	" "	3.7	0.29	35
25	"	C264	37	" "	3.9	0.35	42
26	G	P9	30	" "	5.2	0.05	
27	"	P10	30	" "	5.2	0.05	
28	"	P26	30	" "	4.2	0.09	
29	"	353x85-1	37	" "	4.0	0.29	34
30	"	C264	37	" "	3.7	0.29	28
31	G + trace els.	P9	30	" "	5.3	-0.03	

Table XVII (cont)

The Effect of Trace Elements, Proteose Peptone and Urea on Protein Yield and Cellulose Utilization by Fungi Growing on Newspaper in Medium G

Flask No.	Inoculum Med.	Culture	Temp.	Nutrient Supplement	Term.pH	Protein mg/ml	% Cellulose Utilized
32	G+trace els.	P10	30	Trace els.	5.1	0	
33	"	P26	30	" "	4.8	0	
34	"	353x85-1	37	" "	4.0	0.18	
35	"	C264	37	" "	3.8	0.25	35
36	G+trace els. +proteose peptone	P9	30	" "	3.9	0.18	
37	"	P10	30	" "	4.0	0.18	
38	"	P26	30	" "	4.8	0.03	
39	"	353x85-1	37	" "	4.0	0.25	35
40	"	C264	37	" "	3.8	0.25	35
41	R	P9	30	trace els. and proteose peptone	3.5	0.26	lost
42	"	P10	30	"	3.4	0.30	23
43	"	P26	30	"	3.9	-0.12	
44	"	353x85-1	37	"	3.9	0.14	
45	"	C264	37	"	3.9	0.22	26
46	G	P9	30	"	3.5	0.11	
47	"	P10	30	"	3.4	0.30	23
48	"	P26	30	"	3.6	0.14	
49	"	353x85-1	37	"	3.8	0.18	
50	"	C264	37	"	3.7	0.22	23
51	G + trace els.	P9	30	"	4.0	0.11	
52	"	P10	30	"	3.4	0.30	17
53	"	P26	30	"	3.0	-0.04	
54	"	353x85-1	37	"	3.9	0.11	
55	"	C264	37	"	3.8	0.18	
56	G + trace els. + proteose peptone	P9	30	"	3.3	0.26	26
57	"	P10	30	"	3.3	0.30	35
58	"	P26	30	"	4.2	0	
59	"	353x85-1	37	"	3.8	0.18	
60	"	C264	37	"	3.8	0.18	
61	R	P9	30	Trace els, proteose peptone & urea	5.8	0.29	50

Table XVII (cont)

The Effect of Trace Elements, Proteose Peptone and Urea on Protein Yield and Cellulose Utilization by Fungi Growing on Newspaper in Medium G

Flask No.	Inoculum Med.	Culture	Temp.	Nutrient Supplement	Term. pH	Protein mg/ml	% Cellulose Utilized
62	R	P10	30	Trace els., proteose peptone, and urea	5.7	0.26	lost
63	"	P26	30	"	5.9	-0.08	
64	"	353x85-1	37	"	5.6	0.84	59
65	"	C264	37	"	5.4	0.55	61
66	G	P9	30	"	4.5	0.32	39
67	"	P10	30	"	4.5	0.32	52
68	"	P26	30	"	5.8	-0.04	
69	"	353x85-1	37	"	5.8	0.84	59
70	"	C264	37	"	5.6	0.56	67
71	G+trace els.	P9	30	"	4.4	0.44	43
72	"	P10	30	"	4.6	0.44	50
73	"	P26	30	"	5.6	0	
74	"	353x85-1	37	"	5.3	0.40	65
75	"	C264	37	"	5.3	0.44	70
76	G+trace els. +proteose peptone	P9	30	"	4.9	0.40	59
77	"	P10	30	"	5.0	-0.04	
78	"	P26	30	"	5.0	-0.04	
79	"	353x85-1	37	Trace els., proteose peptone & urea	5.4	0.44	70
80		C264	37	"	5.3	0.62	61
81	Control (none)	-	30	None	4.8	0	0
82	" "	-	30	Trace els.	5.2	0	0
83	" "	-	30	Trace els. & proteose peptone	4.8	0	0
84	" "	-	30	Trace els., proteose peptone & urea	5.0	0	0

Inoculum: 2-day cultures in medium R, medium G, medium G supplemented with "trace elements for medium G" (see Appendix), trace elements plus 0.1% proteose peptone, or trace elements, 0.1% proteose peptone and 0.03% urea, and 2% ball-milled newspaper (Wall St. Journal).

Harvest flasks: Medium G, supplemented with "trace elements for medium G," trace elements plus 0.1% proteose peptone, or trace elements plus 0.1% proteose peptone plus 0.03% urea, and 2% ball-milled newspaper (Wall St. Journal). Harvested in 5 days.

Table XVIII

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

I. Inoculum Cultures

Flask No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml
1	P9	R	30	3.3	0.50
2	P10	"	"	3.3	0.34
3	P26	"	"	4.0	0.14
4	353x85-1	"	37	5.9	0.16
5	C264	"	37	4.3	0.34
6	P9	G	30	4.5	0
7	P10	"	30	4.5	0
8	P26	"	30	3.8	0
9	353x85-1	"	37	3.6	0.04
10	C264	"	37	3.8	0.04
11	P9	G + urea	30	4.6	-0.04
12	P10	"	30	4.6	-0.04
13	P26	"	30	4.4	0
14	353x85-1	"	37	7.3	0.08
15	C264	"	37	6.7	0.08
16	P9	G + urea + trace els.	30	4.5	-0.04
17	P10	"	30	4.5	-0.04
18	P26	"	30	4.3	0
19	353x85-1	"	37	7.2	0.08
20	C264	"	37	6.5	0.08
21	P9	G + urea + trace els. + proteose peptone	30	4.3	0
22	P10	"	30	4.7	0
23	P26	"	30	3.9	0.12
24	353x85-1	"	37	7.3	0.08
25	C264	"	37	6.5	0.20
26	None (control)	R	30	4.9	-
27	" "	G	30	4.6	-
28	" "	G + urea	30	4.6	-
29	" "	G + urea + trace els.	30	4.6	-
30	" "	" + proteose peptone	30	4.6	-

Table XVIII (cont)

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

II. Harvest Cultures

(Inoculated with above Inoculum Cultures)

Flask No.	Inoculum Culture No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml	% Cellulose Consumed
1	1	P9	G	30	3.0	0.16	
2	2	P10	"	30	3.0	0.20	13
3	3	P26	"	30	2.8	0.30	
4	4	353x85-1	"	37	3.3	0.12	
5	5	C264	"	37	3.3	0.12	
6	6	P9	"	30	3.1	0.12	
7	7	P10	"	30	3.0	0.12	
8	8	P26	"	30	2.8	0.22	
9	9	353x85-1	"	37	3.8	0.02	
10	10	C264	"	37	3.3	0.12	
11	11	P9	"	30	3.1	0.20	
12	12	P10	"	30	3.1	0.16	
13	13	P26	"	30	2.9	0.30	
14	14	353x85-1	"	37	3.2	0.16	
15	15	C264	G	37	3.3	0.12	
16	16	P9	"	30	3.0	0.20	
17	17	P10	"	30	3.0	0.20	
18	18	P26	"	30	2.9	0.26	
19	19	353x85-1	"	37	3.2	0.12	
20	20	C264	"	37	3.3	0.12	
21	21	P9	"	30	3.1	0.22	
22	22	P10	"	30	3.1	0.22	
23	23	P26	"	30	2.8	0.33	
24	24	353x85-1	"	37	3.2	0.16	
25	25	C264	"	37	3.3	0.16	
26	1	P9	G+trace els. +proteose peptone	30	3.1	0.30	
27	2	P10	"	30	3.1	0.34	
28	3	P26	"	30	3.3	0.41	
29	4	353x85-1	"	37	3.4	0.16	

Table XVIII (cont)

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

II. Harvest Cultures

(Inoculated with above Inoculum Cultures)

Flask No.	Inoculum Culture No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml	% Cellulose Consumed
30	5	C264	G+trace els. +proteose peptone	37	3.6	0.16	
31	6	P9		30	3.1	0.30	
32	7	P10	"	30	3.0	0.24	
33	8	P26	"	30	3.0	0.22	
34	9	353x85-1	"	37	4.3	0.01	
35	10	C264	"	37	3.5	0.16	
36	11	P9	"	30	3.2	0.34	
37	12	P10	"	30	3.1	0.30	
38	13	P26	"	30	3.0	0.34	
39	14	353x85-1	"	37	3.4	0.22	
40	15	C264	"	37	3.6	0.16	
41	16	P9	"	30	3.2	0.24	
42	17	P10	"	30	3.4	0.30	
43	18	P26	"	30	3.0	0.30	
44	19	353x85-1	"	37	3.4	0.24	
45	20	C264	"	37	3.5	0.18	
46	21	P9	"	30	3.2	0.34	
47	22	P10	"	30	3.1	0.34	
48	23	P26	"	30	3.0	0.41	
49	24	353x85-1	"	37	3.5	0.24	
50	25	C264	"	37	3.6	0.18	
51	1	P9	G+urea	30	4.0	0.44	
52	2	P10	"	30	4.9	0.60	66
53	3	P26	"	30	4.6	0.50	
54	4	353x85-1	"	37	7.2	0.27	19
55	5	C264	"	37	6.7	0.60	
56	6	P9	"	30	4.3	0.42	
57	7	P10	"	30	3.3	0.46	
58	8	P26	"	30	4.7	0.38	
59	9	353x85-1	"	37	5.0	0.38	
60	10	C264	"	37	6.6	0.64	62

Table XVIII (cont)

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

II. Harvest Cultures

(Inoculated with above Inoculum Cultures)

Flask No.	Inoculum Culture No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml	% Cellulose Consumed
61	11	P9	G+urea	30	4.3	0.45	
62	12	P10	"	30	3.5	0.24	
63	13	P26	"	30	7.0	0.34	
64	14	353x85-1	"	37	7.1	-	
65	15	C264	"	37	4.7	0.50	
66	16	P9	"	30	4.2	0.50	
67	17	P10	"	30	4.2	0.34	
68	18	P26	"	30	3.9	0.38	
69	19	353x85-1	"	37	4.8	0.38	
70	20	C264	"	37	7.2	0.38	
71	21	P9	"	30	5.0	0.45	
72	22	P10	"	30	4.4	0.48	
73	23	P26	"	30	3.8	0.34	
74	24	353x85-1	"	37	3.9	0.42	
75	25	C264	"	37	6.3	0.64	62
76	1	P9	G+trace els. + urea	30	5.1	0.48	
77	2	P10	"	30	3.8	0.48	
78	3	P26	"	30	3.9	0.34	
79	4	353x85-1	"	37	5.0	0.34	
80	5	C264	"	37	6.0	0.48	
81	6	P9	"	30	4.2	0.45	
82	7	P10	"	30	3.7	0.16	4
83	8	P26	"	30	3.6	0.34	
84	9	353x85-1	"	37	4.9	0.38	
85	10	C264	"	37	4.8	0.50	
86	11	P9	"	30	3.0	0.45	
87	12	P10	"	30	3.3	0.20	
88	13	P26	"	30	4.6	0.24	
89	14	353x85-1	"	37	6.2	0.30	
90	15	C264	"	37	5.1	0.48	

Table XVIII (cont)

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

II. Harvest Cultures

(Inoculated with above Inoculum Cultures)

Flask No.	Inoculum Culture No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml	% Cellulose Consumed
91	16	P9	G+trace els + urea	30	4.3	0.45	
92	17	P10	"	30	3.7	0.27	
93	18	P26	"	30	5.0	0.2/	
94	19	353x85-1	"	37	4.8	0.34	
95	20	C264	"	37	4.9	0.45	
96	21	P9	"	30	4.7	0.42	
97	22	P10	"	30	4.2	0.45	
98	23	P26	"	30	4.6	0.38	
99	24	353x85-1	"	37	4.8	0.38	
100	25	C264	"	37	4.7	0.42	
101	1	P9	G+trace els +proteose peptone + urea	30	7.0	0.40	
102	2	P10	"	30	5.1	0.48	
103	3	P26	"	30	6.4	0.24	
104	4	353x85-1	"	37	6.1	0.24	
105	5	C264	"	37	6.4	0.34	
106	6	P9	"	30	7.0	0.38	
107	7	P10	"	30	3.7	0.38	
108	8	P26	"	30	3.3	0.70	42
109	9	353x85-1	"	37	7.0	0.05	
110	10	C264	"	37	6.2	0.34	
111	11	P9	"	30	4.2	0.38	
112	12	P10	"	30	4.0	0.38	
113	13	P26	"	30	4.2	0.16	
114	14	353x85-1	"	37	5.2	0.27	
115	15	C264	"	37	6.2	0.40	
116	16	P9	"	30	6.8	0.34	
117	17	P10	"	30	4.8	0.34	
118	18	P26	"	30	4.8	0.24	

Table XVIII (cont)

Further Studies on the Effect of Trace Elements Protopeptone and Urea on Protein Yields from Fungi Growing in Medium G and Reese's Medium

II. Harvest Cultures

(Inoculated with above Inoculum Cultures)

Flask No.	Inoculum Culture No.	Culture	Medium	Temp., °C	Term. pH	Protein mg/ml	% Cellulose Consumed
119	19	353x85-1	G+trace els. +proteose peptone + urea	37	6.6	0.27	
120	20	C264	"	37	5.4	0.38	
121	21	P9	"	30	7.3	0.20	
122	22	P10	"	30	7.0	0.34	
123	23	P26	"	30	6.3	0.24	
124	24	353x85-1	"	37	7.3	0.00	
125	25	C264	"	37	7.5	0.16	
126	Control	None	G	30	4.6	-	0
127	"	None	G+trace els. +proteose peptone	30	4.8	-	
128	"	"	G+urea	30	-	-	0
129	"	"	G+trace els +urea	30	4.6	-	0
130	"	"	G+trace els +proteose peptone +urea	30	4.6	-	0

Inoculum: 2-day cultures in medium R or medium G supplemented as in Part I of the Table with 0.03% urea, trace elements, 0.03% urea and trace elements, trace elements and 0.1% proteose peptone or 0.03% urea trace elements and 0.1% proteose peptone and 2% ball-milled newspaper (Wall St. Journal).

Harvest Flasks: Medium G supplemented as above plus 2% ball-milled newspaper, (Wall St. Journal).

Table XIX

The Comparison of Casamino Acids with Protopeptone as Nutrient Supplements in Reese's Medium and Medium G

Flask No.	Inoculum Grown in Medium *	Culture	Medium	Term. pH	Protein, mg/ml	% Cellulose Consumed
1	RC	P9	R	3.3	0.42	
2	"	P10	R	3.3	0.38	
3	"	P26	"	3.3	0.28	
4	Control	None	"	5.0	-	
5	RC	P9	RC	3.7	0.58	
6	"	P10	"	3.6	0.48	
7	"	P26	"	4.3	0.44	
8	Control	None	"	5.0	-	
9	RC	P9	Med G+trace els.	3.0	0.18	
10	"	P10	"	3.1	0.14	
11	"	P26	"	2.8	0.26	
12	Control	None	"	4.9	-	
13	RC	P9	Med G+trace els.+ urea	6.8	0.62	46
14	"	P10	"	6.8	0.62	43
15	"	P26	"	3.8	0.44	43
16	Control	None	"	4.9	-	-
17	RC	P9	Med G+trace els.+$CaCO_3$	7.7	0.04	
18	"	P10	"	7.1	0.04	
19	"	P26	"	6.7	0.36	
20	Control	None	"	6.5	-	
21	RC	P9	Med G+trace els.+Casamino acids	3.5	0.40	
22	RC	P10	Med G+trace els.+Casamino acids	3.5	0.36	
23	"	P26	"	3.0	0.32	
24	Control	None	"	4.8	-	
25	RC	P9	Med G+trace els.+Casamino acids +urea	7.3	0.34	46
26	"	P10	"	7.4	0.34	36
27	"	P26	"	7.4	0.02	

Table XIX (cont)

The Comparison of Casamino acids with Protopeptone as Nutrient Supplements in Reese's Medium and Medium G

Flask No.	Inoculum Grown in Medium *	Culture	Medium	Term. pH	Protein, mg/ml	% Cellulose Consumed
28	Control	None	Med.G+trace els.+urea + Casamino acids	4.9	-	
29	RC	P9	Med.G+trace els.+urea + proteose peptone	5.6	0.68	51
30	"	P10	"	5.5	0.64	55
31	"	P26	"	7.3	0.06	19
32	Control	None	"	4.9	-	

Inoculum: 2 day cultures in medium R or RC

Harvest Flasks: Medium R or RC, or Medium G supplemented with trace elements, trace elements plus 0.03% urea, trace elements + 1% $CaCO_3$, trace elements + 0.1% casamino acids, trace elements + 0.03% urea + 0.1 % casamino acids, or trace elements + 0.03% urea + 0.1 % proteose peptone. Harvested in 5 days.

Table XX

The Influence of the Ingredients of Reese's Medium on Growth and Protein Yields of Fungi Growing on Newspaper

I. Inoculum Cultures

Flask No.	Culture	Medium	Term. pH	Protein, mg/ml
1	P9	RC	3.3	0.24
2	P10	"	4.3	0.30
3	P26	"	4.9	0.16
4	Control	"	4.7	-
5	P9	RC, using tap water	4.3	0.33
6	P10	"	3.4	0.51
7	P26	"	5.9	0.15
8	Control	"	4.9	-
9	P9	RY	3.9	0.43
10	P10	"	3.3	0.43
11	P26	"	4.9	0.19
12	Control	"	4.8	-
13	P9	RG	3.8	0.26
14	P10	"	3.7	0.38
15	P26	"	5.6	0.08
16	Control	"	4.8	-

II. Harvest Cultures

Flask	Inoc.	Culture.	Media										
			Dist. H_2O	Tap H_2O	KH_2PO_4	$(NH_4)_2SO_4$	Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	CA	Trace Els.	Term. pH	Protein mg/ml	% Cellulose Consumed
1	1	P9	x		x	x	x	x	x	x	x	3.4	0.58	61
2	2	P10	x		x	x	x	x	x	x	x	3.5	0.50	62
3	3	P26	x		x	x	x	x	x	x	x	4.2	0.32	69
4	None	-	x		x	x	x	x	x	x	x	4.7	-	-
5	1	P9		x	x	x	x	x	x	x	x	3.9	0.56	50
6	2	P10		x	x	x	x	x	x	x	x	3.7	0.63	54
7	3	P26		x	x	x	x	x	x	x	x	5.4	0.52	63
8	None	-		x	x	x	x	x	x	x	x	4.9	-	-
9	1	P9		x	x	x	x	x	x	x		3.0	0.50	
10	2	P10		x	x	x	x	x	x	x		5.0	0.00	
11	3	P26		x	x	x	x	x	x	x		4.1	0.58	

Table XX (cont)

II. Harvest Cultures

Flask	Inoc.	Culture	Media										
			Dist. H_2O	Tap H_2O	KH_2PO_4	$(NH_4)_2SO_4$	Urea	$MgSO_4 \cdot 7 H_2O$	$CaCl_2$	CA	Trace Els.	Term. pH	Protein mg/ml	%Cellulose Consumed
12	None	-		x	x	x	x	x	x	x		5.0	-	
13	1	P9		x	x	x	x	x	x		x	3.2	0.42	
14	2	P10		x	x	x	x	x	x		x	4.8	0.00	
15	3	P26		x	x	x	x	x	x		x	3.4	0.36	
16	None	-		x	x	x	x	x	x		x	4.8	-	
17	1	P9		x	x	x	x	x		x	x	4.2	0.65	50
18	2	P10		x	x	x	x	x		x	x	3.8	0.58	
19	3	P26		x	x	x	x	x		x	x	5.2	0.50	
20	None	-		x	x	x	x	x		x	x	4.9	-	
21	1	P9		x	x	x	x		x	x	x	4.2	0.65	60
22	2	P10		x	x	x	x		x	x	x	4.0	0.65	
23	3	P26		x	x	x	x		x	x	x	6.3	0.50	
24	None	-		x	x	x	x		x	x	x	5.0	-	
25	1	P9		x	x	x		x	x	x	x	2.9	0.43	
26	2	P10		x	x	x		x	x	x	x	5.0	0.00	
27	3	P26		x	x	x		x	x	x	x	2.6	0.54	
28	None	-		x	x	x		x	x	x	x	4.9	-	
29	1	P9		x	x		x	x	x	x	x	6.3	0.50	
30	2	P10		x	x		x	x	x	x	x	5.2	0.47	
31	3	P26		x	x		x	x	x	x	x	6.8	0.28	
32	None	-		x	x		x	x	x	x	x	5.1	-	
33	1	P9		x	x	x	x	x		x		3.6	0.48	44
34	2	P10		x	x	x	x	x		x		3.6	0.58	51
35	3	P26		x	x	x	x	x		x		3.5	0.48	60
36	None	-		x	x	x	x	x		x		4.7	-	
37	1	P9		x	x	x	x			x		3.5	0.70	74
38	2	P10		x	x	x	x			x		3.2	0.85	71
39	3	P26		x	x	x	x			x		3.2	0.85	74
40	None	-		x	x	x	x			x		5.0	-	
41	1	P9		x	x	x				x		2.8	0.47	
42	2	P10		x	x	x				x		2.8	0.32	
43	3	P26		x	x	x				x		2.5	0.72	
44	None	-		x	x	x				x		5.0	-	

Table XX (cont)

II. Harvest Cultures

Flask	Inoc.	Culture	Media										
			Dist. H_2O	Tap H_2O	KH_2PO_4	$(NH_4)_2SO_4$	Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	CA	Trace Els.	Term. pH	Protein mg/ml	%Cellulose Consumed
45	1	P9		x	x						x	4.7	0.14	
46	2	P10		x	x						x	4.7	0.10	
47	3	P26		x	x						x	4.9	0.10	
48	None	-		x	x						x	5.0	-	
49	1	P9		x							x	4.3	0.07	
50	2	P10		x							x	4.3	0.18	
51	3	P26		x							x	8.0	0.02	
52	None	-		x							x	4.9	-	
53	1	P9		x	x	x	x	x	x			3.2	0.62	
54	2	P10		x	x	x	x	x	x			3.3	0.36	
55	3	P26		x	x	x	x	x	x			3.3	0.322	
56	None	-		x	x	x	x	x	x			4.9	-	
57	1	P9		x	x	x	x					2.8	0.23	
58	2	P10		x	x	x	x					2.9	0.16	
59	3	P26		x	x	x	x					2.6	0.30	
60	None	-		x	x	x	x					4.8	-	

Inoculum: 2 day cultures in medium RC plus 2% ball-milled Wall Street Journal

Harvest Flasks: Medium R with 2% ball-milled newspaper (Wall St. Journal) supplemented according to Part II of the Table. Harvested in 5 days.

Table XXI

Growth and Protein Yields from Fungi Growing on Ball-Milled Wall Street Journal vs. Ball Milled Denver Post in Medium G 8

Flask No.	Inoculum Grown in	Culture	Paper	Term.pH	Protein mg/ml	% Cellulose Consumed
1	RC+WSJ	P9	WSJ	6.7	0.54	67
2	"	P9	"	4.0	0.59	48
3	"	P10	"	5.0	0.63	60
4	"	P10	"	4.9	0.59	67
5	"	P26	"	3.6	0.52	58
6	"	P26	"	3.5	0.48	61
7	RC+Denver Post	P9	DP	5.0	0.74	61
8	"	P9	"	4.3	0.67	50
9	"	P10	"	6.0	0.63	63
10	"	P10	"	4.1	0.63	53
11	"	P26	"	3.9	0.45	46
12	"	P26	"	4.1	0.41	46
13	Control	None	WSJ	4.5	-	0
14	Control	None	DP	4.7	-	0

Inoculum: 2 day cultures in medium RC and either 2% ball-milled Wall Street Journal or 2% ball-milled Denver Post.

Harvest Flasks: Medium G8 and either 2% ball-milled Wall Street Journal or 2% ball-milled Denver Post. Harvested in 5 days.

Table XXII

Growth and Protein Yields from Fungi Grown on Ball-Milled Wall Street Journal Heated at 200°C for 25 Minutes vs. Unheated in Med.G-8

Flask No.	Inoculum Medium	Culture	Paper	Term.pH	Protein mg/ml	% Cellulose Consumed
1	RC	P9	Unheated	7.3	0.59	57
2	"	P10	"	5.7	0.63	65
3	"	P26	"	7.6	0.13	13
4	"	None	"	4.5	-	-
5	"	P9	Heated	4.3	0.68	68
6	"	P10	"	4.4	0.68	75
7	"	P26	"	3.4	0.62	62
8	"	None	"	4.1	-	-

Inoculum: 2 day cultures in medium RC and 2% ball-milled unheated Wall Street Journal.

Harvest Flasks: Medium G-8 and either heated or unheated 2% ball-milled Wall Street Journal. Harvested in 5 days.

Table XXIII

Two-Day Protein Yields from Fungus Cultures Grown on Reese's Medium Supplemented with Disodium Ethylenediamine tetraacetic Acid in Place of Protopeptone and 2% Ball-milled Newspaper

Flask No.	Culture	Medium	Term. pH	Protein, mg/ml
1	P9	R	3.1	0.58
2	P10	"	2.9	0.54
3	P26	"	3.1	0.25
4	Control	"	4.7	-
5	P9	R+0.1% EDTA less protopeptone	3.4	0.16
6	P10	"	3.3	0.16
7	P26	"	4.0	0.06
8	Control	"	4.9	-
9	P9	R+0.01% EDTA less protopeptone	2.9	0.09
10	P10	"	2.9	0.07
11	P26	"	3.1	0.04
12	Control	"	4.7	-

Inoculum: 1 ml of a suspension from an agar slant culture in 5 ml of distilled water added to each fermentation flask.

Table XXIV

Comparative Yields of Protein and Cellulose Utilization in Five Days from Reese's Medium and Medium G Modified by Omitting Ingredients and Substituting Other Ingredients

Flask No.	Culture	Distilled Water	Tap Water	KH_2PO_4	$(NH_4)_2SO_4$	0.03% Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Casamino Acids	Trace els. for Reese's Medium	$(NH_4)_2HPO_4$	Yeast Autolysate	Trace els. for Med.G	Urea, 6%	Media Base	Term. pH	Protein, mg/ml	%Cellulose Utilized
1	P9	x		x	x	x	x	x	x	x					Reese	3.3	0.54	47
2	"	x		x	x	x	x	x	x	x					"	3.4	0.54	47
3	P10	x		x	x	x	x	x	x	x					"	3.4	0.56	45
4	"	x		x	x	x	x	x	x	x					"	3.4	0.56	58
5	P26	x		x	x	x	x	x	x	x					"	3.2	0.60	54
6	"	x		x	x	x	x	x	x	x					"	3.4	0.56	56
7	-	x		x	x	x	x	x	x	x					"	4.7	-	-
8	P9		x	x	x	x	x	x	x	x					"	3.4	0.60	62
9	"		x	x	x	x	x	x	x	x					"	3.5	0.64	48
10	P10		x	x	x	x	x	x	x	x					"	3.4	0.56	54
11	"		x	x	x	x	x	x	x	x					"	3.6	0.56	57
12	P26		x	x	x	x	x	x	x	x					"	3.5	0.68	57
13	"		x	x	x	x	x	x	x	x					"	3.7	0.54	61
14	-		x	x	x	x	x	x	x	x					"	5.0	-	-
15	P9	x		x	x	x			x						"	3.0		
16	"	x		x	x	x			x						"	3.3	0.54	
17	P10	x		x	x	x			x						"	3.4	0.51	
18	"	x		x	x	x			x						"	3.5	0.58	
19	P26	x		x	x	x			x						"	3.2	0.50	
20	"	x		x	x	x			x						"	2.9	0.76	
21	-	x		x	x	x			x						"	4.7	-	
22	P9		x	x	x	x			x						"	3.5	0.55	
23	"		x	x	x	x			x						"	3.5	0.59	
24	P10		x	x	x	x			x						"	3.5	0.57	
25	"		x	x	x									x	"	3.4	0.44	
26	P26		x	x	x	x			x						"	3.6	0.49	
27	"		x	x	x	x			x						"	3.6	0.59	
28	-		x	x	x	x			x						"	4.8	-	
29	P9	x		x	x	x	x	x		x		x			"	3.1	0.45	
30	"	x		x	x	x	x	x		x		x			"	3.0	0.50	
31	P10	x		x	x	x	x	x		x		x			"	3.0	0.54	

Table XXIV

Comparative Yields of Protein and Cellulose Utilization in Five Days from Reese's Medium and Medium G Modified by Omitting Ingredients and Substituting Other Ingredients

Flask No.	Culture	Distilled Water	Tap Water	KH_2PO_4	$(NH_4)_2SO_4$	0.03% Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Casamino Acids	Trace els. for Reese's Medium	$(NH_4)_2HPO_4$	Yeast Autolysate	Trace els. for Med.G	Urea, 6%	Media Base	Term. pH	Protein, mg/ml	%Cellulose Utilized
32	P10	x		x	x	x	x	x		x		x			Reese	3.1	0.55	
33	P26	x		x	x	x	x	x		x		x			"	3.4	0.44	
34	P26	x		x	x	x	x	x		x		x			"	3.5	-	
35	-	x		x	x	x	x	x		x		x			"	4.7	-	
36	P9		x	x	x	x	x	x		x		x			"	3.2	0.54	41
37	"		x	x	x	x	x	x		x		x			"	3.1	0.56	36
38	P10		x	x	x	x	x	x		x		x			"	3.1	0.60	36
39	"		x	x	x	x	x	x		x		x			"	3.1	0.60	85
40	P26		x	x	x	x	x	x		x		x			"	3.5	0.40	53
41	"		x	x	x	x	x	x		x		x			"	3.6	-	58
42	-		x	x	x	x	x	x		x		x			"	5.2	-	-
43	P9	x		x	x	x						x			"	3.4	0.50	
44	"	x		x	x	x						x			"	2.9	0.32	
45	P10	x		x	x	x						x			"	3.4	0.42	
46	"	x		x	x	x						x			"	3.4	0.42	
47	"	x		x	x	x						x			"	3.5	0.46	
48	"	x		x	x	x						x			"	3.5	0.32	
49	-	x		x	x	x						x			"	5.0	-	
50	P9		x	x	x	x						x			"	3.2	0.47	29
51	P9		x	x	x	x						x			"	2.9	0.32	0
52	P10		x	x	x	x						x			"	3.2	0.53	59
53	"		x	x	x	x						x			"	3.3	0.53	21
54	P26		x	x	x	x						x			"	3.4	0.49	50
55	"		x	x	x	x						x			"	3.3	0.49	0
56	-		x	x	x	x						x			"	5.0	-	-
57	P9	x				x			x		x				G	7.4	0.43	
58	"	x				x			x		x				"	7.3	0.46	
59	P10	x				x			x		x				"	7.4	0.04	
60	"	x				x			x		x				"	7.0	0.50	
61	P26	x				x			x		x				"	7.7	0.00	
62	"	x				x			x		x				"	7.6	0.00	
63	-	x				x			x		x				"	4.7	-	

Table XXIV

Comparative Yields of Protein and Cellulose Utilization in Five Days from Reese's Medium and Medium G Modified by Omitting Ingredients and Substituting Other Ingredients

	Flask No.	Culture	Distilled Water	Tap Water	KH_2PO_4	$(NH_4)_2SO_4$	0.03% Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Casamino Acids	Trace els. for Reese's Medium	$(NH_4)_2HPO_4$	Yeast Autolysate	Trace els. for Med.G	Urea, 6%	Media Base	Term. pH	Protein, mg/ml	%Cellulose Utilized
	64	P9		x			x			x		x				G	5.4	0.78	
	65	"		x			x			x		x				"	7.2	0.40	
	66	P10		x			x			x		x				"	6.8	0.61	
	67	"		x			x			x		x				"	6.2	0.72	
	68	P26		x			x			x		x				"	7.5	0.00	
	69	"		x			x			x		x				"	7.6	0.02	
	70	-		x			x			x		x				"	4.7	-	
	71	P9	x				x			x		x		x		"	5.9	0.64	
	72	"	x				x			x		x		x		"	7.0	0.50	
*	73	P10	x				x			x		x		x		"	7.3	0.04	
*	74	"	x				x			x		x		x		"	7.4	0.08	
*	75	P26	x				x			x		x		x		"	5.1	0.04	
*	76	"	x				x			x		x		x		"	7.0	0.00	
	77	-	x				x			x		x		x		"	4.6	-	
*	78	P9		x			x			x		x		x		"	7.3	0.00	
*	79	"		x			x			x		x		x		"	7.4	0.06	
*	80	P10		x			x			x		x		x		"	7.3	0.00	
*	81	"		x			x			x		x		x		"	7.5	0.08	
*	82	P26		x			x			x		x		x		"	6.4	0.06	
*	83	"		x			x			x		x		x		"	7.0	0.00	
	84	-		x			x			x		x		x		"	4.8	-	
	85	P9	x				x					x	x			"	3.9	0.58	
*	86	"	x				x					x	x			"	5.4	0.58	
*	87	P10	x				x					x	x			"	5.3	0.58	
*	88	"	x				x					x	x			"	4.9	0.64	
*	89	P26	x				x					x	x			"	6.6	0.00	
*	90	"	x				x					x	x			"	7.1	0.00	
	91	-	x				x					x	x			"	4.8	-	
*	92	P9		x			x					x	x			"	7.4	0.03	
*	93	"		x			x					x	x			"	7.6	0.03	
*	94	P10		x			x					x	x			"	7.5	0.03	
*	95	"		x			x					x	x			"	7.5	0.00	

Table XXIV

Comparative Yields of Protein and Cellulose Utilization in Five Days from Reese's Medium and Medium G Modified by Omitting Ingredients and Substituting Other Ingredients

	Flask No.	Culture	Distilled Water	Tap Water	KH_2PO_4	$(NH_4)_2SO_4$	0.03% Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Casamino Acids	Trace els. for Reese's Medium	$(NH_4)_2HPO_4$	Yeast Autolysate	Trace els. for Med.G	Urea,.6%	Media Base	Term.pH	Protein, mg/ml	%Cellulose Utilized
	96	P26		x			x					x	x			G	7.5	0.00	
	97	"		x			x					x	x			"	7.5	0.00	
	98	-		x			x					x	x			"	4.8	-	
	99	P9	x				x					x	x	x		"	4.1	0.71	31
*	100	"	x				x					x	x	x		"	5.7	0.61	55
*	101	P10	x				x					x	x	x		"	5.7	0.57	64
*	102	"	x				x					x	x	x		"	5.5	-	81
	103	P26	x				x					x	x	x		"	7.1	0.15	9
	104	"	x				x					x	x	x		"	7.4	0.03	0
	105	-	x				x					x	x	x		"	4.7	-	-
*	106	P9		x			x					x	x	x		"	7.5	0.00	15
*	107	"		x			x					x	x	x		"	4.6	0.64	68
	108	P10		x			x					x	x	x		"	4.0	0.70	64
	109	"		x			x					x	x	x		"	4.0	0.76	50
	110	P26		x			x					x	x	x		"	3.6	0.74	50
	111	"		x			x					x	x	x		"	7.5	0.08	12
	112	-		x			x					x	x	x		"	4.9	-	-
	113	P9	x		x	x							x		x	Reese	4.7	0.62	
	114	"	x		x	x							x		x	"	4.6	0.58	
	115	P10	x		x	x							x		x	"	4.2	0.58	
	116	"	x		x	x							x		x	"	5.1	0.59	
	117	P26	x		x	x							x		x	"	7.0	0.18	
	118	"	x		x	x							x		x	"	5.2	0.70	
	119	-	x		x	x							x		x	"	5.1	-	
	120	P9		x	x	x							x		x	"	4.5	0.55	
	121	"		x	x	x							x		x	"	4.3	0.55	
	122	P10		x	x	x							x		x	"	4.8	0.55	
	123	"		x	x	x							x		x	"	4.8	0.55	
	124	P26		x	x	x							x		x	"	7.2	0.32	
	125	"		x	x	x							x		x	"	5.5	0.61	
	126	-		x	x	x							x		x	"	5.2	-	
	127	P9	x									x	x		x	G	7.5	0.30	

Table XXIV

Comparative Yields of Protein and Cellulose Utilization in Five Days from Reese's Medium and Medium G Modified by Omitting Ingredients and Substituting Other Ingredients

Flask No.	Culture	Distilled Water	Tap Water	KH_2PO_4	$(NH_4)_2SO_4$	0.03% Urea	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Casamino Acids	Trace els. for Reese's Medium	$(NH_4)_2HPO_4$	Yeast Autolysate	Trace els. for Med.G	Urea.6%	Media Base	Term.pH	Protein, mg/ml	%Cellulose Utilized
128	P9	x									x	x		x	G	7.6	0.30	
129	P10	x									x	x		x	"	7.5	0.44	
130	"	x									x	x		x	"	7.5	0.42	
131	P26	x									x	x		x	"	7.8	0.03	
132	"	x									x	x		x	"	7.7	0.03	
133	-	x									x	x		x	"	4.8	-	
134	P9		x								x	x		x	"	7.5	0.24	
135	"		x								x	x		x	"	7.6	0.23	
136	P10		x								x	x		x	"	7.5	0.42	
137	"		x								x	x		x	"	7.5	-	
138	P26		x								x	x		x	"	7.7	0.00	
139	"		x								x	x		x	"	7.7	0.00	
140	-		x								x	x		x	"	4.8	-	

* Flasks incubated at 37°C rather than 30°C.

Inoculum - 2 day culture grown in medium RC.

Table XXV

The Influence of Shaking Rate on Protein Yields from Cultures Growing on Ball-Milled Newspaper in Reese's Medium Supplemented with Casamino Acids Instead of Protopeptone (RC).

Flask No.	Culture	Shaking Rate, RPM	pH	Protein mg/ml
1	P9	350	3.1	lost
2	"	"	4.0	0.45
3	P10	"	3.9	0.45
4	"	"	4.1	0.52
5	P26	"	3.5	0.48
6	"	"	3.5	0.45
7	Control	"	4.8	-
8	P9	175	4.2	0.57
9	"	"	3.9	0.54
10	P10	"	4.1	0.50
11	"	"	4.5	0.54
12	P26	"	3.8	0.54
13	"	"	3.7	0.54
14	Control	"	4.8	-
15	P9	264	4.3	0.56
16	"	"	4.2	0.52
17	P10	"	3.8	0.59
18	"	"	4.0	0.52
19	P26	"	3.4	0.59
20	"	"	3.3	0.62
21	Control	"	4.8	-

Inoculum: 2 day cultures in medium RC with 2% ball-milled newspaper (Denver Post).

Harvest: 5 day cultures in the same medium.

Table XXVI

The Influence of Paper and Nitrogen Concentration on Protein Yields from Fungi Growing in Modified Medium G

Flask No.	Culture	Medium Supplement	Paper, g/100 ml	Term. pH	Protein mg/ml
1	P9	None	2	3.5	0.28
2	"	"	4	3.0	0.55
3	"	"	8	2.8	0.68
4	"	"	16	2.8	0.48
5	P10	"	2	3.5	0.45
6	"	"	4	2.9	0.61
7	"	"	8	2.8	0.68
8	"	"	16	2.8	0.68
9	P26	"	2	4.0	0.39
10	"	"	4	2.7	0.78
11	"	"	8	2.8	0.78
12	"	"	16	2.9	0.58
13	Control	"	2	5.0	-
14	"	"	4	4.8	-
15	"	"	8	4.6	-
16	"	"	16	4.4	-
17	P9	10xthe usual Nitrogen	2	3.7	0.58
18	"	"	4	3.2	0.79
19	"	"	8	3.0	0.79
20	"	"	16	2.8	1.32
21	P10	"	2	3.7	0.32
22	"	"	4	3.1	0.52
23	P10	"	8	3.0	0.50
24	"	"	16	2.8	1.24
25	P26	"	2	4.0	-
26	"	"	4	3.2	0.67
27	"	"	8	2.8	0.91
28	"	"	16	2.8	1.34
29	Control	"	2	4.9	-
30	"	"	4	4.8	-
31	"	"	8	4.5	-
32	"	"	16	4.4	-

Inoculum: 2-day cultures in medium RC with 2% ball-milled newspaper (Denver Post)
Harvest: 5-day cultures in Medium RY plus different amts. of ball-milled " "

Table XXVII

The Influence of Paper and Nitrogen Concentration on Protein Yields from Fungi in Modified Medium G

Flask No.	Culture	Paper g/100 ml	$(NH_4)_2HPO_4$ g/100 ml	Urea g/100 ml	Nutrient Supplement	pH	Protein mg/ml
1	P9	2	1	0.03	Cas.acids	6.7	0.02
2	"	4	"	"	"	7.0	0.60
3	"	8	"	"	"	3.5	1.30
4	"	16	"	"	"	3.0	1.94
5	"	2	"	"	Yeast Autolysate	6.9	0.58
6	"	4	"	"	"	4.0	0.85
7	"	8	"	"	"	3.2	0.89
8	"	16	"	"	"	2.7	1.34
9	"	2	"	"	None	6.8	0.43
10	"	4	"	"	"	3.9	0.67
11	"	8	"	"	"	3.2	0.88
12	"	16	"	"	"	2.9	1.16
13	Control	2	"	"	Cas.acids	4.8	-
14	"	4	"	"	"	4.5	-
15	"	8	"	"	"	4.3	-
16	"	16	"	"	"	4.2	-
17	"	2	"	"	Yeast Autolysate	4.7	-
18	"	4	"	"	"	4.5	-
19	"	8	"	"	"	4.3	-
20	"	16	"	"	"	4.2	-
21	"	2	"	"	None	4.5	-
22	"	4	"	"	"	4.3	-
23	Control	8	1	0.03	None	4.3	-
24	"	16	"	"	"	4.1	-
25	P9	2	5	"	Cas.acids	6.4	0.30
26	P9	4	"	"	"	4.1	0.43
27	"	8	"	"	"	6.2	0.43
28	"	16	"	"	"	5.6	0.02
29	"	2	"	"	Yeast Autolysate	5.5	0.62
30	"	4	"	"	"	3.8	0.92
31	"	8	"	"	"	3.3	0.52
32	"	16	"	"	"	4.2	0.26

Table XXVII (cont)

The Influence of Paper and Nitrogen Concentration on Protein Yields from Fungi in Modified Medium G

Flask No.	Culture	Paper g/100 ml	$(NH_4)_2HPO_4$ g/100 ml	Urea g/100ml	Nutrient Supplement	pH	Protein mg/ml
33	P9	2	5	0.03	None	5.6	0.44
34	"	4	"	"	"	3.5	0.17
35	"	8	"	"	"	3.4	0.87
36	"	16	"	"	"	4.1	0.12
37	Control	2	"	"	Cas.acids	4.5	-
38	"	4	"	"	"	4.4	-
39	"	8	"	"	"	4.3	-
40	"	16	"	"	"	4.2	-
41	"	2	"	"	Yeast Autolysate	4.5	-
42	"	4	"	"	"	4.3	-
43	"	8	"	"	"	4.3	-
44	"	16	"	"	"	4.1	-
45	"	2	"	"	None	4.3	-
46	"	4	"	"	"	4.3	-
47	"	8	"	"	"	4.2	-
48	"	16	"	"	"	4.1	-
1	P10	2	1	0.03	Cas.acids	6.9	0.48
2	"	4	"	"	"	6.8	0.92
3	"	8	"	"	"	3.4	1.28
4	"	16	"	"	"	3.2	2.08
5	"	2	"	"	Yeast Autolysate	7.0	0.63
6	"	4	"	"	"	5.2	1.17
7	"	8	"	"	"	3.2	1.20
8	"	16	"	"	"	2.9	0.84
9	"	2	"	"	None	7.0	0.64
10	"	4	"	"	"	4.1	0.81
11	"	8	"	"	"	3.1	0.48
12	"	16	"	"	"	3.1	0.96
13	P26	2	"	"	Cas.acids	6.6	0.14
14	"	4	"	"	"	5.3	0.28
15	"	8	"	"	"	2.6	1.08
16	"	16	"	"	"	2.8	1.28
17	"	2	"	"	Yeast Autolysate	3.2	0.63
18	"	4	"	"	"	2.7	0.85

Table XXVII (cont)

The Influence of Paper and Nitrogen Concentration on Protein Yields from Fungi in Modified Medium G

Flask No.	Culture	Paper g/100ml	$(NH_4)_2HPO_4$ g/100 ml	Urea g/100ml	Nutrient Supplement	pH	Protein mg/ml
19	P26	8	1	0.03	Yeast Autolysate	2.7	0.96
20	"	16	"	"	"	2.5	0.22
21	"	2	"	"	None	2.9	0.56
22	"	4	"	"	"	2.9	0.66
23	"	8	"	"	"	2.8	0.90
24	"	16	"	"	"	2.6	1.46
25	Control	2	1	0.03	Cas.acids	4.7	-
26	"	4	"	"	"	4.6	-
27	"	8	"	"	"	4.5	-
28	"	16	"	"	"	4.3	-
29	"	2	"	"	Yeast Autolysate	4.7	-
30	"	4	"	"	"	4.6	-
31	"	8	"	"	"	4.4	-
32	"	16	"	"	"	4.3	-
33	"	2	"	"	None	4.5	-
34	"	4	"	"	"	4.4	-
35	"	8	"	"	"	4.3	-
36	"	16	"	"	"	4.3	-
37	P10	2	5	"	Cas.acids	6.3	0.32
38	"	4	"	"	"	6.1	0.00
39	"	8	"	"	"	6.0	0.00
40	"	16	"	"	"	6.0	0.22
41	"	2	"	"	Yeast Autolysate	6.2	0.34
42	"	4	"	"	"	3.7	0.67
43	"	8	"	"	"	3.9	0.21
44	"	16	"	"	"	5.5	0.02
45	"	2	"	"	None	5.5	0.65
46	"	4	"	"	"	5.7	0.58
47	"	8	"	"	"	5.6	0.40
48	"	16	"	"	"	4.5	0.00
49	P26	2	5	0.03	Cas.acids	6.6	0.51
50	"	4	"	"	"	6.5	0.10

Table XXVII (cont)

The Influence of Paper and Nitrogen Concentration on Protein Yields from Fungi in Modified Medium G

Flask No.	Culture	Paper g/100 ml	$(NH_4)_2HPO_4$ g/100 ml	Urea g/100ml	Nutrient Supplement	pH	Protein mg/ml
51	P26	8	5	0.03	Cas.acids	3.3	0.19
52	"	16	"	"	"	3.0	0.94
53	"	2	"	"	Yeast Autolysate	6.4	0.22
54	"	4	"	"	"	5.9	0.54
55	"	8	"	"	"	3.2	0.63
56	"	16	"	"	"	2.8	1.66
57	"	2	"	"	None	5.7	0.31
58	"	4	"	"	"	4.6	0.28
59	"	8	"	"	"	3.0	0.52
60	"	16	"	"	"	2.6	1.34
61	Control	2	"	"	Cas.acids	4.7	-
62	"	4	"	"	"	4.5	-
63	"	8	"	"	"	4.4	-
64	"	16	"	"	"	4.1	-
65	"	2	"	"	Yeast Autolysate	4.4	-
66	"	4	"	"	"	4.4	-
67	"	8	"	"	"	4.3	-
68	"	16	"	"	"	4.2	-
69	"	2	"	"	None	4.5	-
70	"	4	"	"	"	4.3	-
71	"	8	"	"	"	4.2	-
72	"	16	"	"	"	4.0	-

Inoculum: 3-day cultures medium RY with 2% ball-milled newspaper, (Denver Post)

Harvest: 14-day cultures in Medium G, medium G-1, medium G-5 and medium G-6 plus different amounts of ball-milled newspaper (Denver Post)

Table XXVIII

The Effect of Tween-80 on Growth and Protein Production by Fungi Growing on Ball-Milled Newspaper in Modified Reese's Medium & Medium G

Flask No.	Culture	Medium	Tween-80, mg/ml	pH	Protein, mg/ml
1	P9	RY	0.00	5.2	0.29
2	"	"	0.01	4.2	0.44
3	"	"	0.10	3.7	0.59
4	"	"	0.50	3.5	0.48
5	"	G	0.00	6.5	0.65
6	"	"	0.01	6.5	0.63
7	"	"	0.10	4.0	0.67
8	"	"	0.50	6.8	0.28
9	P10	RY	0.00	3.8	0.29
10	"	"	0.01	3.9	0.51
11	"	"	0.10	3.6	0.66
12	"	"	0.50	3.4	0.54
13	"	G	0.00	5.3	0.73
14	"	"	0.01	5.6	0.67
15	"	"	0.10	5.2	0.64
16	"	"	0.50	-	-
17	P26	RY	0.00	3.4	0.52
18	"	"	0.01	3.3	0.57
19	"	"	0.10	3.3	0.66
20	"	"	0.50	3.1	0.64
21	"	G	0.00	3.7	0.47
22	"	"	0.01	4.0	0.50
23	"	"	0.10	3.4	0.64
24	"	"	0.50	3.7	0.64
25	Control	RY	0.00	4.6	-
26	"	"	0.01	4.8	-
27	"	"	0.10	4.8	-
28	"	"	0.50	4.8	-
29	"	G	0.00	4.5	-
30	"	"	0.01	4.5	-
31	"	"	0.10	4.5	-
32	"	"	0.50	4.5	-

Inoculum: 2-day cultures in Medium RY with 2% ball-milled newspaper (Denver Post)

Harvest: 5-day cultures in medium RY or in medium G, both with 2% ball-milled newspaper (Denver Post).

Table XXIX

Protein Yields from Fungi Growing on Coarsely Shredded Newspaper vs. Ball-Milled Newspaper in Medium G Supplemented with Extra Nutrients

Flask No.	Culture	Supplement	Paper+	Harvest time, days	Term. pH	Protein mg/ml
1	P9	Urea	BM	5	4.3	0.56
2	P10	"	"	"	3.8	0.54
3	P26	"	"	"	3.3	0.35
4	P9	"	S	"	5.6	0.23
5	P10	"	"	"	5.3	0.16
6	P26	"	"	"	7.3	0.00
7	P9	"	SH	"	5.8	0.20
8	P10	"	"	"	5.9	0.06
9	P26	"	"	"	5.0	0.04
10	Control	"	BM	"	4.5	-
11	"	"	S	"	4.3	-
12	"	"	SH	"	4.4	-
13	P9	Urea and Yeast autol.	BM	"	4.0	0.65
14	P10	"	"	"	3.7	0.54
15	P26	"	"	"	7.3	0.00
16	P9	"	S	"	7.5	0.19
17	P10	"	"	"	7.4	0.10
18	P26	"	"	"	7.0	0.00
19	P9	"	SH	"	7.2	0.07
20	P10	"	SH	5	7.0	0.04
21	P26	"	"	"	6.2	0.03
22	Control	"	BM	"	4.6	-
23	"	"	S	"	4.4	-
24	"	"	SH	"	4.4	-
1	P9	Urea	BM	5	7.7	0.32
2	P10	"	"	"	5.0	0.63
3	P26	"	"	"	3.1	0.24
4	P9	"	S	"	7.0	0.22
5	P10	"	"	"	4.9	0.08
6	P26	"	"	"	7.7	0.02
7	P9	"	SH	"	6.9	0.08
8	P10	"	"	"	7.0	0.06

Table XXIX (cont)

Protein Yields from Fungi Growing on Coarsely Shredded Newspaper *vs.* Ball-Milled Newspaper in Medium G Supplemented with Extra Nutrients

Flask No.	Culture	Supplement	Paper[+]	Harvest time, days	Term. pH	Protein mg/ml
9	P26	Urea	SH	5	4.5	0.06
10	Control	"	BM	"	4.5	-
11	"	"	S	"	4.4	-
12	"	"	SH	"	4.2	-
13	P9	Urea + yeast autolysate	BM	"	4.1	1.00
14	P10	"	"	"	4.4	0.98
15	P26	"	"	"	7.6	0.08
16	P9	"	S	"	7.6	0.04
17	P10	"	S	"	7.6	0.04
18	P26	"	"	"	7.6	0.00
19	P9	"	SH	"	7.4	0.05
20	P10	"	"	"	7.3	0.19
21	P26	"	SH	5	5.1	0.08
22	Control	"	BM	"	4.6	-
23	"	"	S	"	4.6	-
24	"	"	SH	"	4.2	-
25	P9	Urea	BM	9	3.1	1.31
26	P10	"	"	"	3.5	0.63
27	P26	"	"	"	6.8	0.13
28	P9	"	S	"	7.2	0.00
29	P10	"	"	"	7.1	0.09
30	P26	"	"	"	7.3	0.07
31	P9	"	SH	"	7.0	0.02
32	P10	"	"	"	6.4	0.00
33	P26	"	"	"	5.7	0.00
34	Control	"	BM	"	4.4	-
35	"	"	S	"	4.4	-
36	"	"	SH	"	4.1	-
37	P9	Urea + yeast autolysate	BM	"	4.8	0.58
38	P10	"	"	"	6.6	0.63
39	P26	"	"	"	2.9	0.76
40	P9	"	S	"	7.3	0.09

Table XXIX (cont)

Protein Yields from Fungi Growing on Coarsely Shredded Newspaper vs. Ball-Milled Newspaper in Medium G Supplemented with Extra Nutrients

Flask No.	Culture	Supplement	Paper+	Harvest time, days	Term. pH	Protein mg/ml
41	P10	Urea + yeast autolysate	S	9	7.2	0.00
42	P26	"	"	"	7.4	0.00
43	P9	"	SH	"	7.2	0.00
44	P10	"	"	"	7.1	0.00
45	P26	"	"	"	6.6	0.55
46	Control	"	BM	"	4.6	-
47	"	"	S	"	4.5	-
48	"	"	SH	"	4.3	-
49	P9	Urea	BM	14	6.8	0.56
50	P10	"	"	"	6.6	0.61
51	P26	"	"	"	3.9	0.51
52	P9	"	S	"	6.8	0.28
53	P10	"	"	"	6.9	0.30
54	P26	"	"	"	7.0	0.15
55	P9	"	SH	"	6.8	0.29
56	P10	"	"	"	6.7	0.24
57	P26	"	"	"	3.7	0.53
58	Control	"	BM	"	4.6	-
59	"	"	S	"	4.5	-
60	"	"	SH	"	4.3	-
61	P9	Urea + yeast autolysate	BM	"	6.6	0.59
62	P10	"	"	"	6.9	0.59
63	P26	"	"	"	2.9	0.67
64	P9	"	S	"	6.9	0.20
65	P10	"	"	"	6.6	0.13
66	P26	"	"	"	6.3	0.17
67	P9	"	SH	"	6.6	0.00
68	P10	"	"	"	7.1	0.00
69	P26	"	"	"	6.5	0.16
70	Control	"	BM	"	4.7	-
71	"	"	S	"	4.6	-
72	"	"	SH	"	-	-

+ Paper - BM=ball-milled, S=shredded, SH=shredded and heated to 200 C for 15 min.

Inoculum: 2-day cultures in medium RY with ball-milled newspaper + 2% Denver Post

Harvest: Media G1 and G3 harvested in 5,9 and 14 days.

Table XXX

Stirred-Jar Fermentor Studies with Myrothecium verrucaria

Grown on Newspaper

Run No.	Fermentor No.	Medium	Paper[4]	Initial pH	Term. pH	Inc. time, days	Stirring rate RPM	Aeration rate, l/min	Inoculum	Protein yield mg/ml	% Cellulose Utilised
3	1	F,10 l	NEW,2%	5.3	3.25[1]	9	100	2.4	R,200ml	0.48	46
	2	G,10 l	NEW,2%	5.3	4.90	9	100	2.4	R,200ml	0.51	67
	3	H,10 l	NEW,2%	5.3	4.15	9	100	2.4	R,200ml	0.44	62
4	1	G,10 l	NEW,2%	5.3	3.45	12	0	2.4	R,200ml	0.42	59
	2	G,10 l	NEW,2%	5.3	4.65[2]	12	0	2.4	R,200ml	0.47	62
	3	G,10 l	NEW,2%	5.2	6.50	12	0	2.4	R,200ml	0.35	64
5	1	G,10 l	NEW,2%	5.3	4.00	14	0	2.4	4-200ml[3]	0.44	58
	2	G,10 l	NEW,2%	5.3	4.35[2]	14	0	2.4	4-200ml	0.48	52
	3	G,10 l	NEW,2%	6.5	6.40[2]	14	0	2.4	4-200ml	0.30	54
6	1	G-1,10 l	NEW,2%	5.3	5.50	21	0	2.4	R,200ml	0.22	63
	2	G-1,10 l	NEW,4%	5.3	4.95	21	0	2.4	R,200ml	0.57	57
	3	G-2,10 l	NEW,2%	5.3	5.20	21	0	2.4	R,200ml	0.25	57
7	1	G-3,10 l	BMD,2%	5.3	5.75	24	100	2.4	RY,200ml	0.25	77
	2	G-3,10 l	BMD,4%	5.3	5.30	24	100	2.4	RY,200ml	0.34	74
	3	G-3, 6.5? l	BMD,8%	5.3	5.40	24	100	2.4	RY,200ml	0.63	45
8	1	G-1,10 l	BMD,2%	5.3	5.65	20	100	2.4	RY,200ml	0.25	70
	2	G-1,10 l	NEW,2%	5.3	5.55	20	100	2.4	RY,200ml	0.40	81
	3	G-3,10 l	BMD,2%	5.3	5.80	20	100	2.4	RY,200ml	0.36	77
9	1	G-3,10 l	BMD,4%	5.3	5.65	18	100	1.5	RY,200ml	0.33	34
	2	G-3,10 l	BMD,4%	5.3	5.55	18	100	3.0	RY,200ml	0.47	46
	3	G-3,10 l	BMD,4%	5.3	5.35	18	100	6.0	RY,200ml	0.94	61
10	1	G-3,10 l	BMD,4%	5.3	4.50	14	300-400[5]	1.5	RY,100ml	1.19	63
	2	G-3,10 l	BMD,4%	5.3	4.50	14	300-400	3.0	RY,100ml	1.27	65
	3	G-3,10 l	BMD,4%	5.3	4.55	14	300-400	6.0	RY,100ml	1.36	63
11	1	G-3,10 l	BMD,4%	5.3	4.15	11	400	3.0	RY,200ml	1.19	68
	2	G-3,10 l	BMD,4%	5.3	4.10	11	400	3.0	RY,200ml	0.97	63
	3	G-3,10 l	BMD,4%	5.3	3.60	11	400	3.0	RY,200ml	1.02	61
12	1	G-3,7.5 l	BMD,4%	5.3	4.05	12	400	3.0	RY,150ml	1.42	62
	2	G-4,7.5 l	BMD,4%	5.3	4.15	12	400	3.0	RY,150ml	1.11	51
	3	G-1,715 l	BMD,4%	5.3	4.40	12	400	3.0	RY,150ml	0.89	52

Table XXX

Footnotes:

1. pH was adjusted on day 7 to 5.3 by adding sterile 1 N NaOH. Prior to this point pH values, never having been adjusted, were:
   1 3.05
   2 3.55
   3 3.25
2. These samples were pH-controlled by automatic titration. See Figure 7
3. These inocula consisted of 200 ml of culture from run no. 4, refrigerated until use. Jar 1 was inoculated from jar 1 of run 4, jar 2 from jar 2, and jar 3 from jar 3.
4. Paper BMW = ball-milled Wall Street Journal
   BMD = ball-milled Denver Post
   The amount given as % is grams of paper per 100 ml of culture medium.
5. The higher stirring rate of run 10 resulted in some foaming losses and build-up of solids on the walls of the fermentor above the normal liquid level. Antifoam, (Hodag FD-82, diluted to contain 9 g/l) was therefore used in runs 11 and 12.

Table XXXI

Analytical Data on the Dried Ground Harvest from Stirred-Jar 3, Run No. 10

	% (By Weight)		
	Harvest	Corn	Wheat
Moisture Content	2.4, 2.5	10.6	10.5
Ash Content	14, 13	1.53	1.8
Biuret, Total Protein	2.8, 2.9	-	-
Biuret, Soluble Protein	2.1, 2.0	-	-
Ammonia Nitrogen	4.60, 4.50, 4.53	-	-
Organic Nitrogen	1.01, 1.05	-	-
"Protein"*	6.31, 6.57	10.3	11.9
Total Kjeldahl Nitrogen	5.61, 5.58	-	-
Nitrate Nitrogen	< 0.0005, < 0.0005	-	-
Nitrite Nitrogen	0.0001, 0.0001	-	-
Total Carbohydrates	29, 31	-	-
Soluble Carbohydrates	0.99, 0.93	-	-
Cellulose Content	23, 22	2.2**	1.8**
Total Lipids	2.2, 2.3	5.0	2.1

* Organic Nitrogen x 6.25

** "Fiber" (cellulose + lignin)

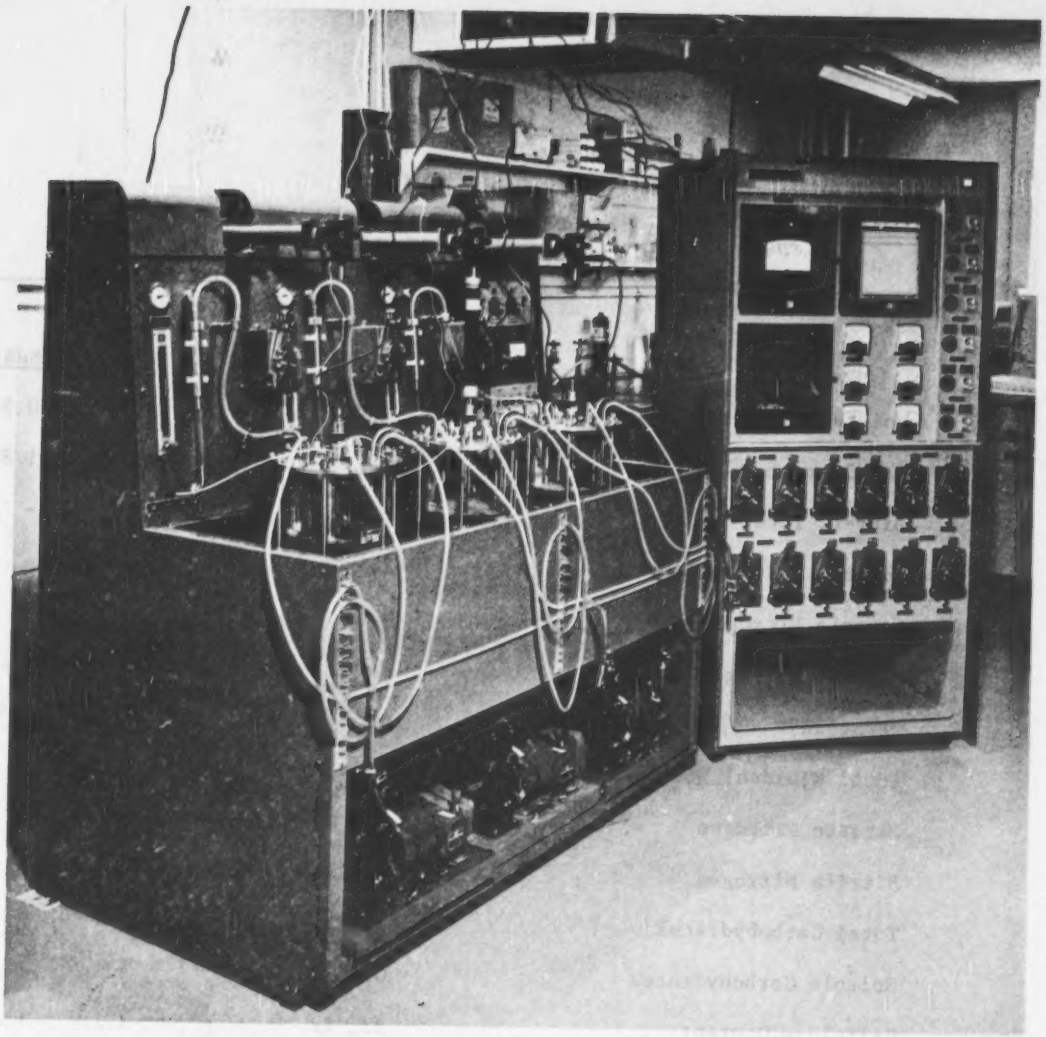

Figure 1. Stirred-Jar Apparatus in Use for Fermentation of Ball-Milled Newspaper

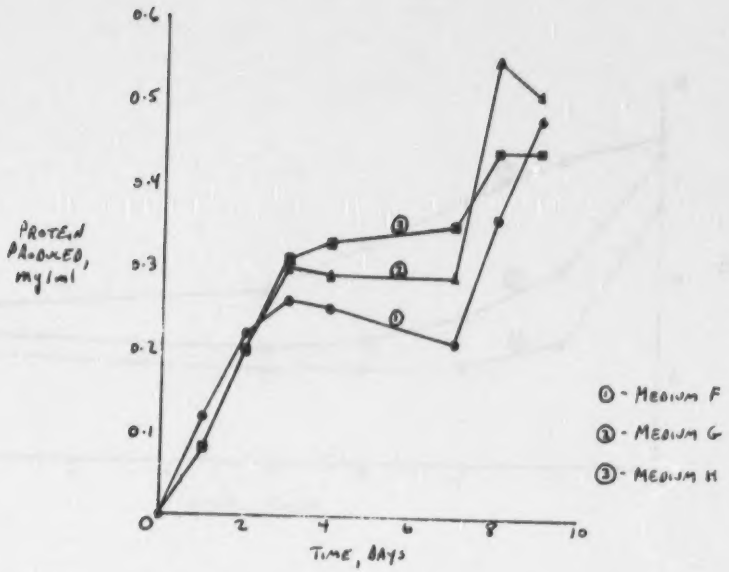

Figure 2. Protein Production, Stirred-Jar Run 3

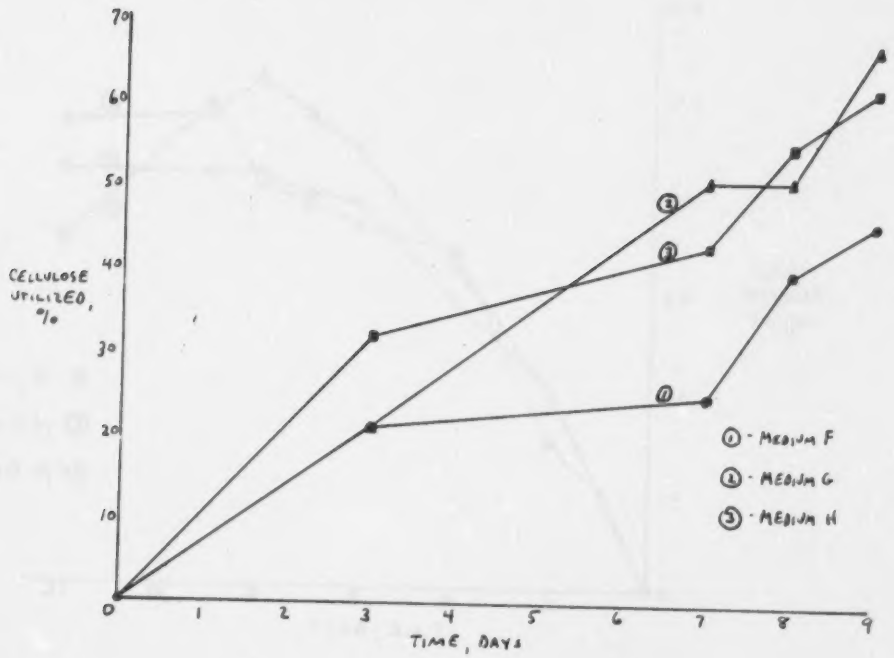

Figure 3. Cellulose Utilization, Stirred-Jar Run 3

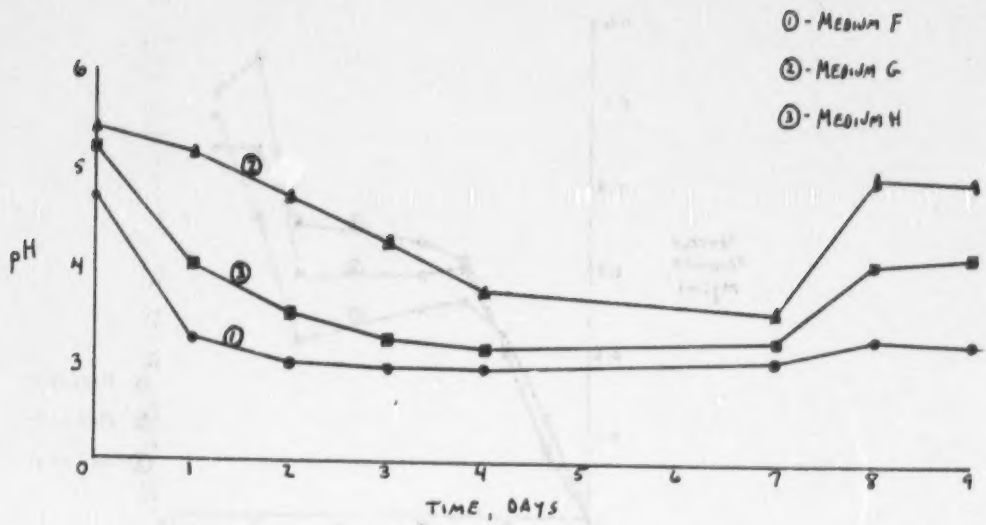

Figure 4. Variation in pH, Stirred-Jar Run 3

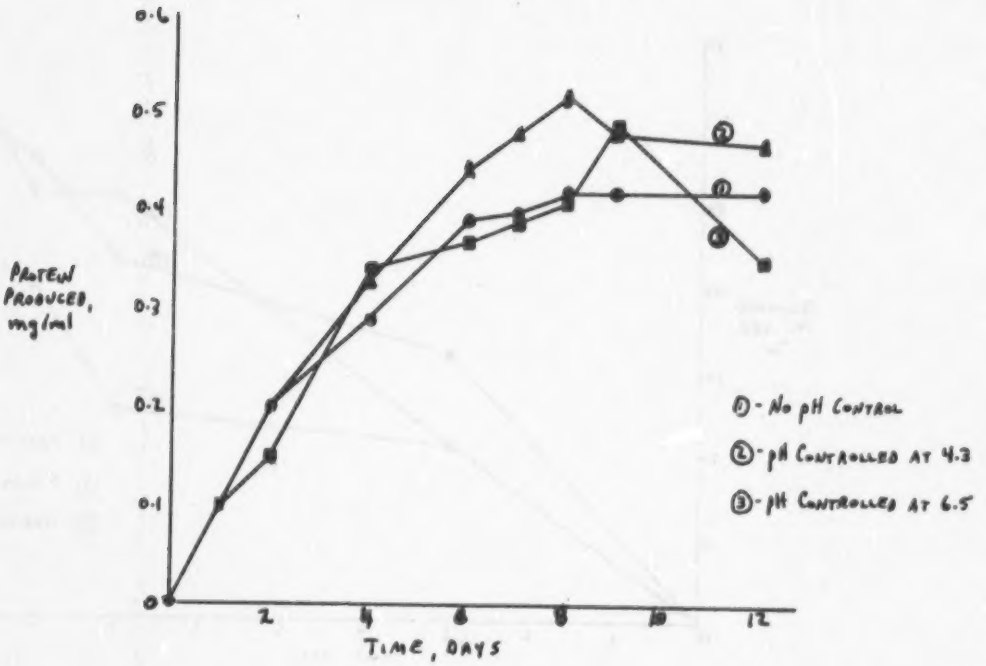

Figure 5. Protein Production, Stirred-Jar Run 4

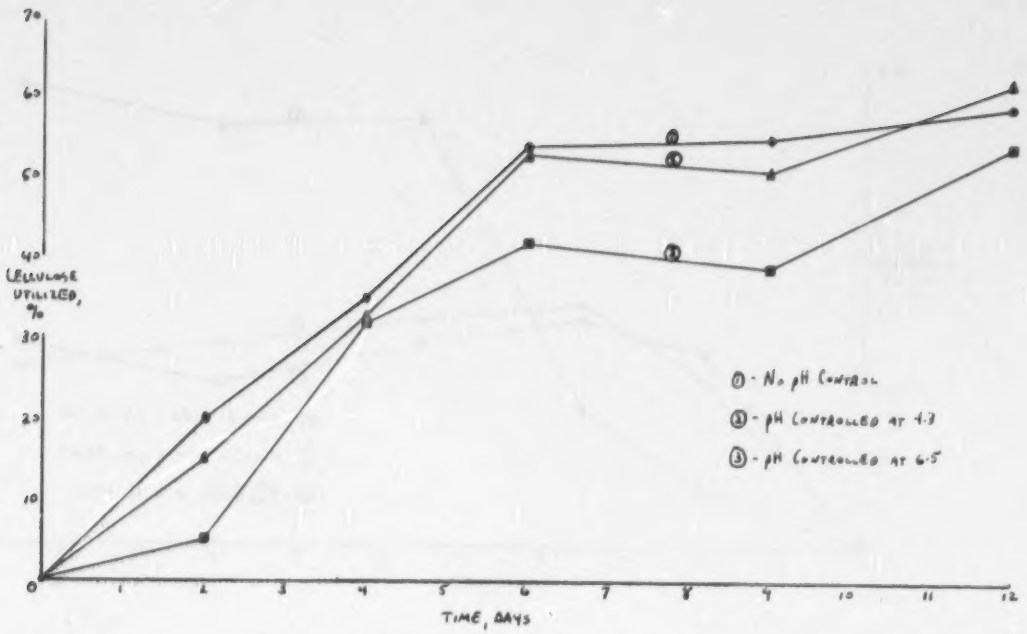

Figure 6. Cellulose Utilization, Stirred-Jar Run 4

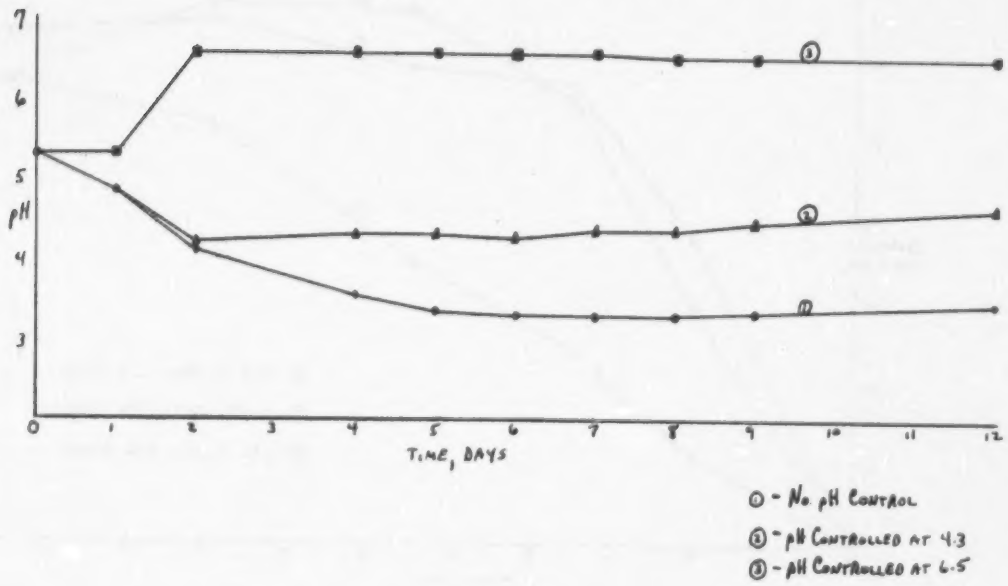

Figure 7. Variation in pH, Stirred-Jar Run 4

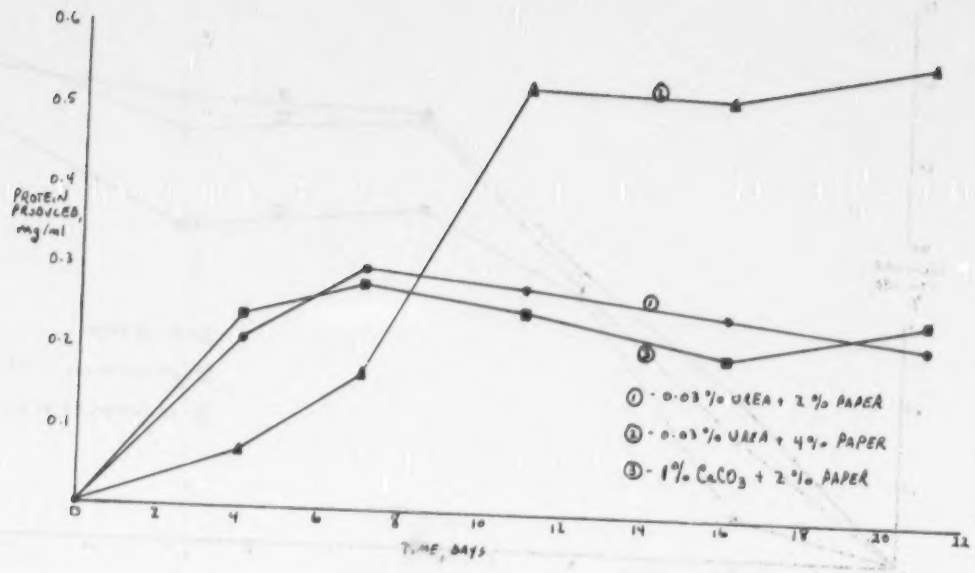

Figure 8. Protein Production, Stirred-Jar Run 6

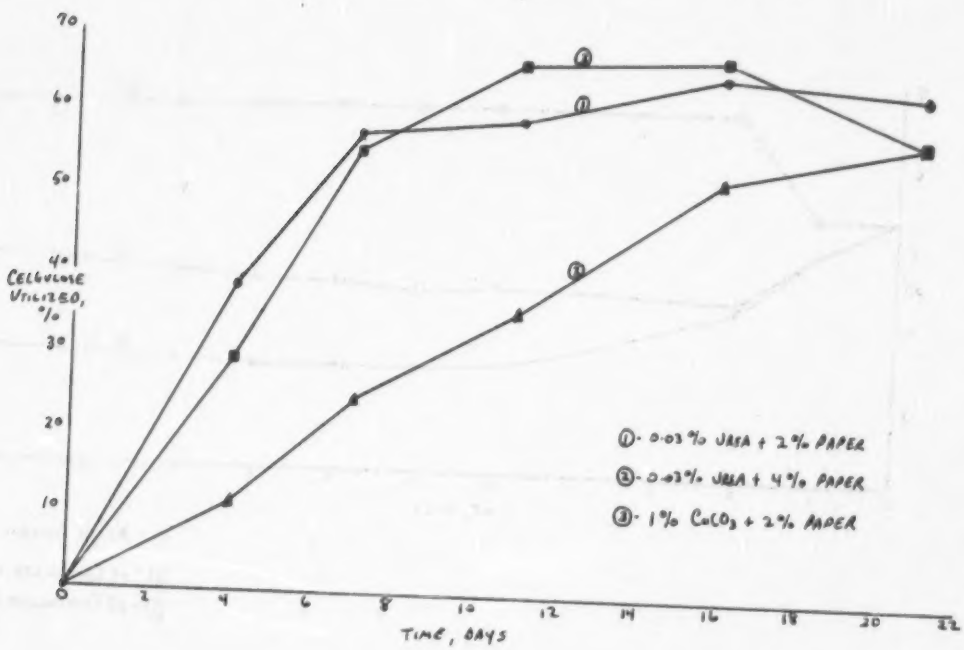

Figure 9. Cellulose Utilization, Stirred-Jar Run 6

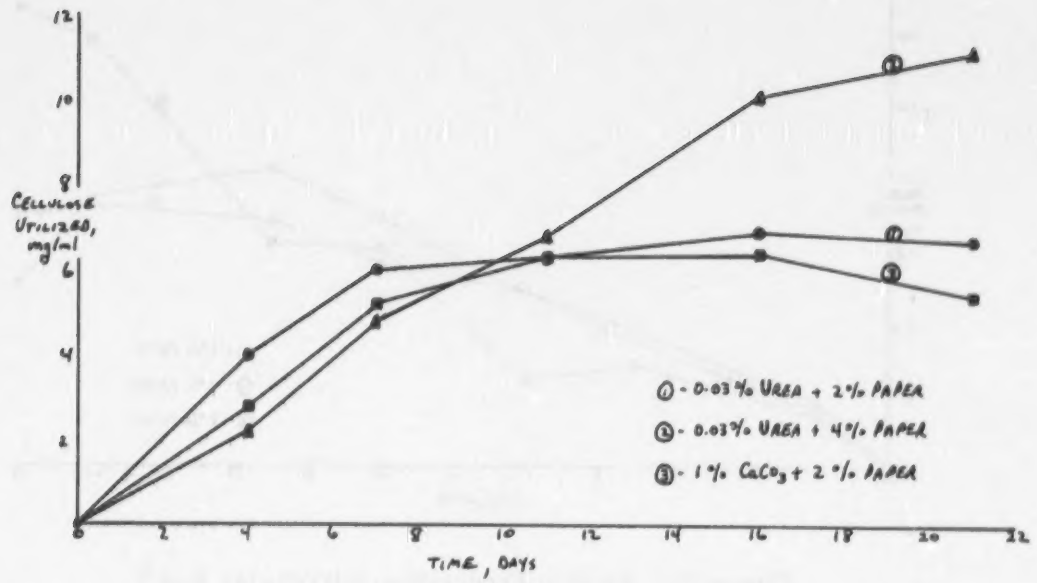

Figure 10. Cellulose Utilization, Stirred-Jar Run 6

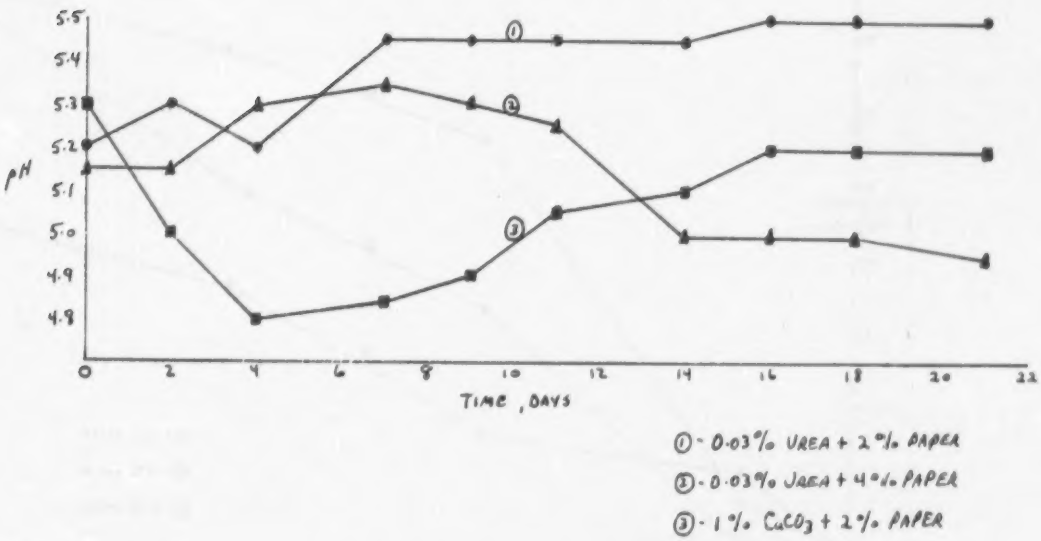

Figure 11. Variation in pH, Stirred-Jar Run 6

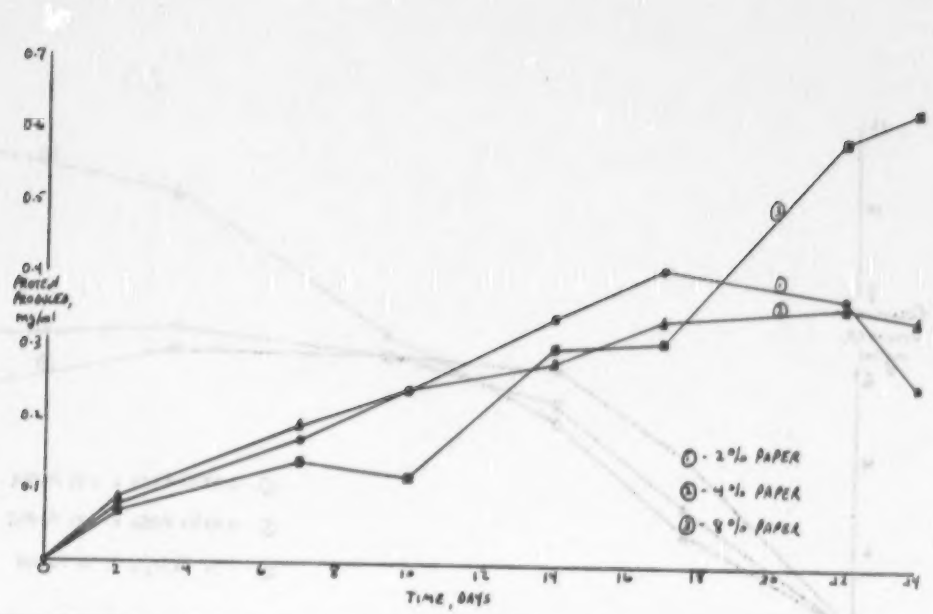

Figure 12. Protein Production, Stirred-Jar Run 7

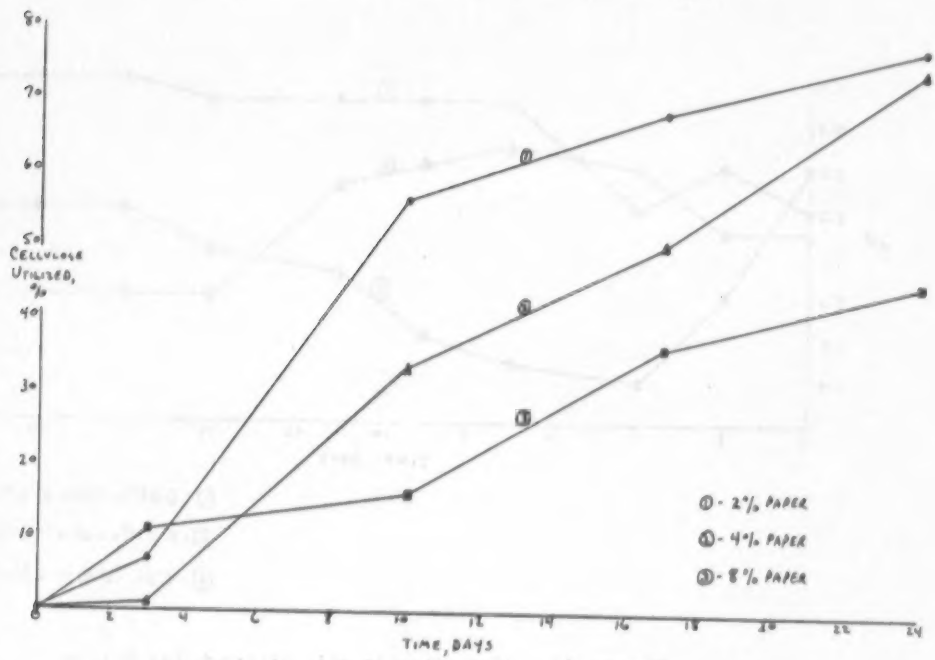

Figure 13. Cellulose Utilization, Stirred-Jar Run 7

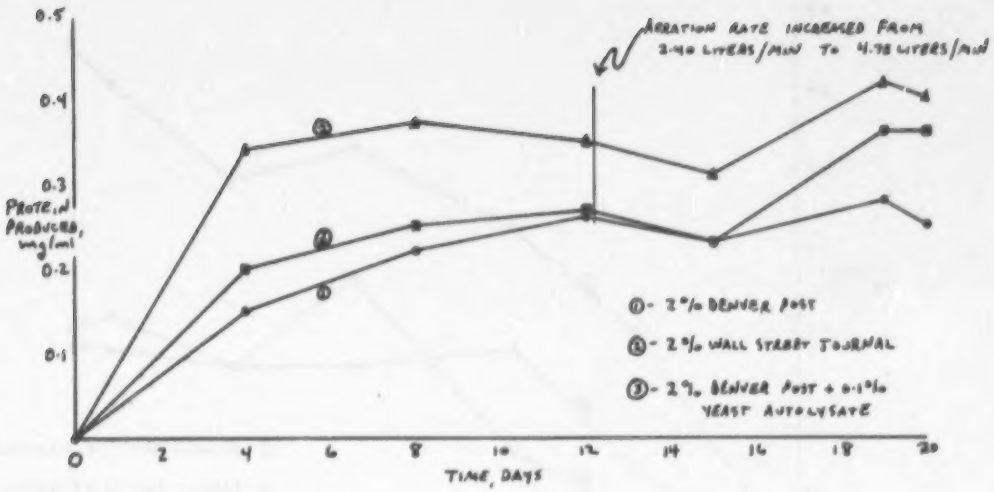

Figure 14. Protein Production, Stirred-Jar Run 8

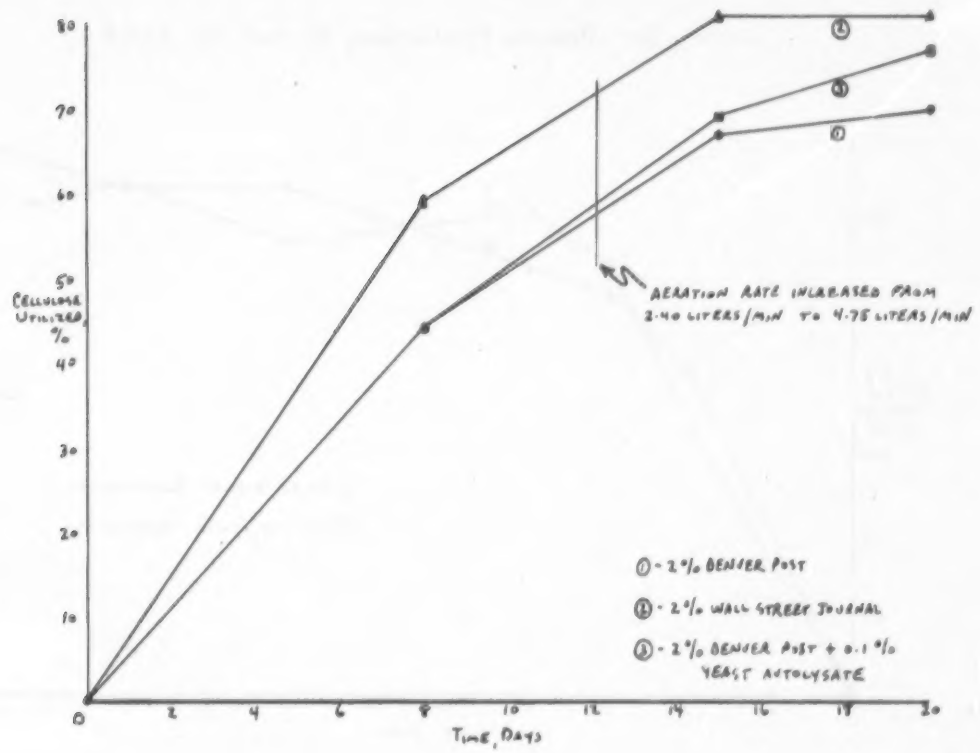

Figure 15. Cellulose Utilization, Stirred-Jar Run 8

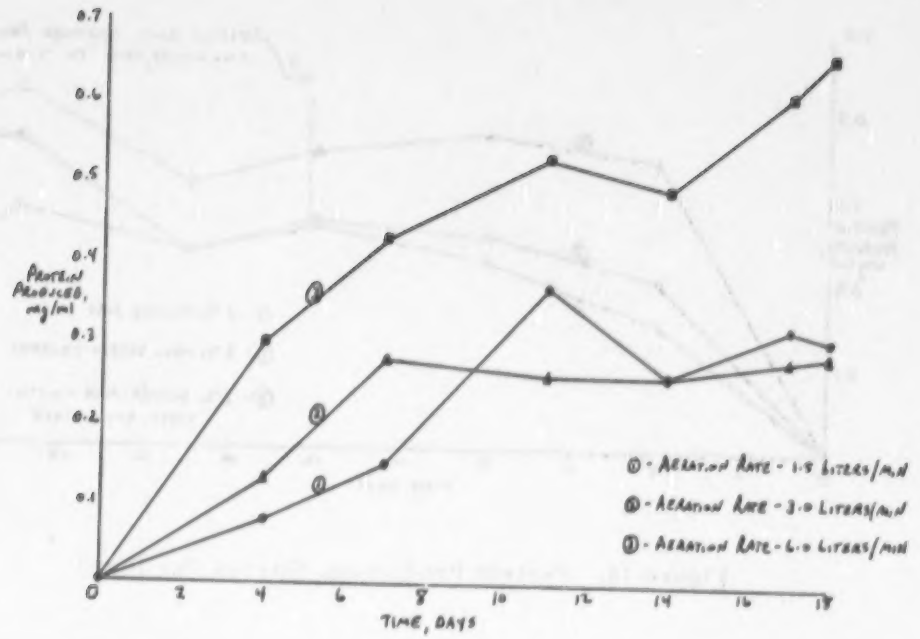

Figure 16. Protein Production, Stirred-Jar Run 9

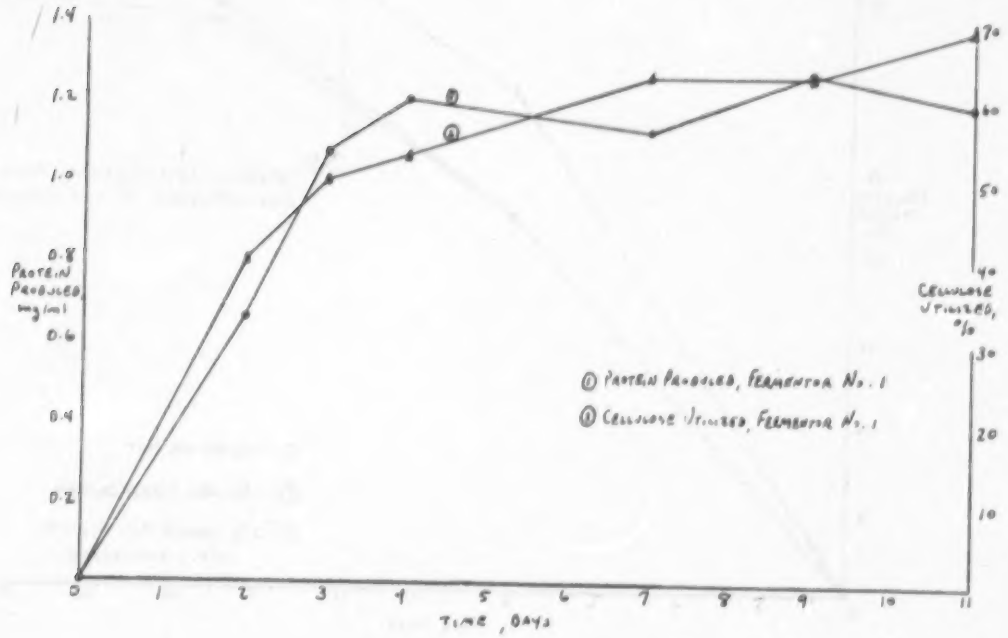

Figure 17. Protein Production and Cellulose Utilization, Stirred-Jar Run 11

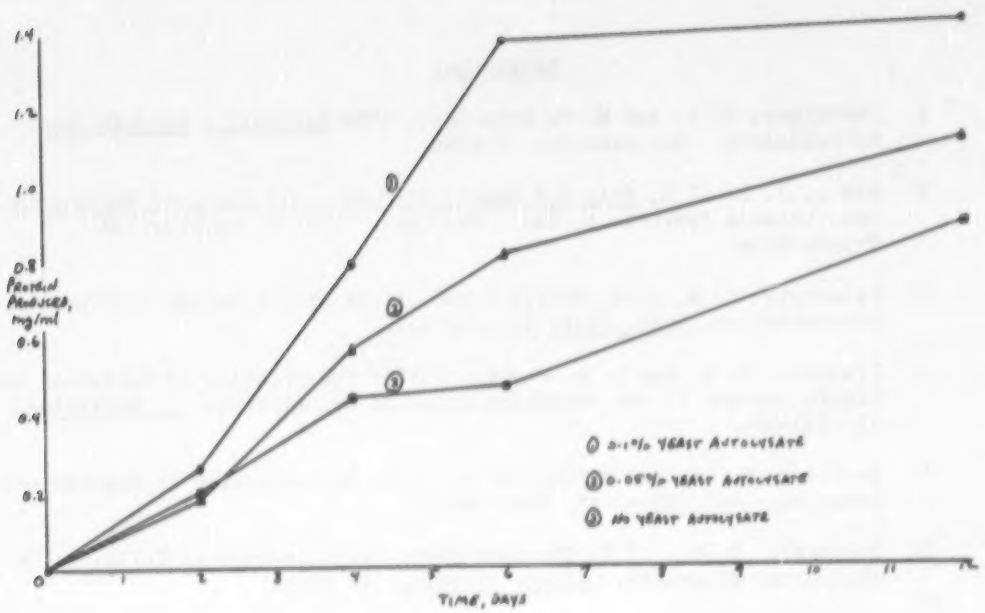

Figure 18. Protein Produced, Stirred-Jar Run 12

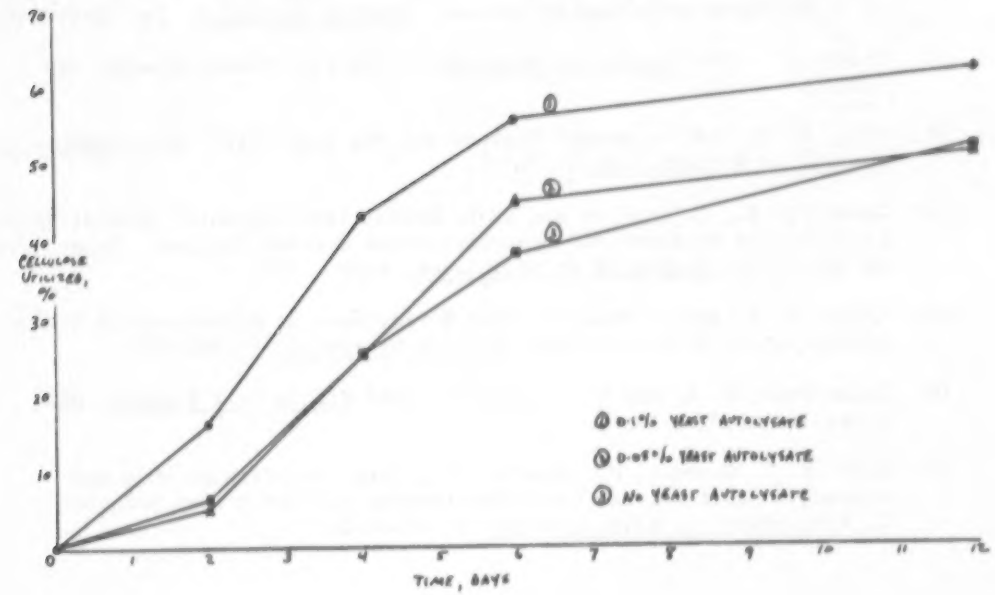

Figure 19. Cellulose Utilized, Stirred-Jar Run 12

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Publications Supported by Grant

1. Updegraff, D. M., 1970, The Semi-Micro Determination of Cellulose in Biological Materials, Analytical Biochem. In Press.

Staffing

1. Dr. John D. Douros, Principal Investigator, June 1, 1967 - July 15, 1968. 50% of time.

2. Dr. David M. Updegraff, Principal Investigator, July 15, 1968 to date. 50% of time.

3. Dr. Thomas D. Nevens, Senior Research Engineer, June 1, 1967 to date. 15% of time.

4. Miss Sandra Hockersmith, Microbiologist Specialist, June 1, 1967 to Jan. 1, 1969, 50% of time.

5. Miss L. Myra Fuller, B.S. Chemist, Scientist C., June 1, 1967 to May, 1968. 50% of time.

6. Mrs. Diane Mack, B.S., Microbiologist, on project as a technician under Miss Hockersmith, June 1967 to Dec. 1968. Since then in charge of fermentations microbiology laboratory, until her departure in May 1969.

7. Mr. Larry Griffin, Technician, carried out all of the 14 liter stirred-jar fermentor work, and most of the chemical analytical work on the product.

8. Mrs. Jeannette King, B.S., Microbiologist, worked in the fermentation laboratory during the entire period of the project.

9. Mrs. Sharon Zaun, M.S. Biologist, worked in fermentation laboratory from May 1968 to August 1969.

Appendix

Table I

Culture Media Used for Isolation of Hydrocarbon-Oxidizing Organisms and Some Cellulose Utilizers

Medium	Concentration g/l distilled H_2O
Medium 1	
$CaCl_2 \cdot 2H_2O$	.15
KCl	.40
$MgSO_4 \cdot 7H_2O$	2.0
NaH_2PO_4	.9
Na_2HPO_4	2.0
$NaNO_3$	20.0
1 ml of microelements (Salts B)	
Salicylic Acid Medium (pH=7)	
$(NH_4)_2SO_4$	2.5
$MgSO_4$	.5
$MnCl_2 \cdot 4H_2O$	.2
$FeCl_2 \cdot 4H_2O$	.05
$CaCl_2$	.05
K_2HPO_4	1.0
KH_2PO_4	.5
Raymonds Medium (pH=7)	
$MgSO_4 \cdot 7H_2O$	0.2
KH_2PO_4	0.4
Na_2HPO_4	0.6
Na_2CO_3	0.1
$(NH_4)_2SO_4$	1.0
$CaCl_2$	0.1
$MnSO_4$	0.002
$FeSO_4 \cdot 7H_2O$	0.005

Table I (cont.)

Medium	Concentration g/l distilled H_2O
Medium 2	
Ammonium phosphate (Dibasic)	10.0
Potassium phosphate (Dibasic)	5.0
Sodium sulfate	0.5
Salts "B" Microelements #4	10 cc
Microelements Medium 4 (Salts "B")	
$MgSO_4 \cdot 7H_2O$	40.0
$FeSO_4 \cdot 7H_2O$	2.0
$MnSO_4 \cdot 4H_2O$	2.0
NaCl	2.0
Yeast Hydrocarbon Medium (pH=6.5)	g/liter tap water
$Na_2HPO_4 \cdot 12H_2O$	2.5
KH_2PO_4	3.5
$MgSO_4 \cdot 7H_2O$	1.0
NaCl	0.5
Urea or NH_4NO_3	5.0
Yeast Medium of British Petroleum (pH=4)	g/liter distilled H_2O
$(NH_4)_2HPO_4$	2.0
KCl	1.15
$MgSO_4 \cdot 7H_2O$	0.65
$ZnSO_4 \cdot H_2O$	0.17
$MnSO_4 \cdot 10H_2O$	0.045
$FeSO_4 \cdot 7H_2O$	0.068
Yeast Extract	0.025
PM Medium	
$CaCl_2$	0.01
$FeSO_4 \cdot 7H_2O$	0.1
85% H_3PO_4	1.5
KCl	0.5
$MgSO_4$	0.5
$MnSO_4$	0.11
Na_2SO_4	0.1
NH_4Cl	0.11

Table II

Culture Media Used Primarily for Cellulolytic Microorganisms

Bushnell and Haas Medium (pH=7.0 to 7.2)	Concentration g/l distilled H_2O
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.02
KH_2PO_4	1.0
K_2HPO_4	1.0
NH_4NO_3	1.0
$FeCl_3 4H_2O$	2 drops
Modified Czapeks Medium (pH=4.0)	
$NaNO_3$	3
K_2HPO_4	1
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.05
Carbon Source	20.0
Srinivasan's Medium (pH=6.3) (SM)	
NaCl	6.0
$(NH_4)_2SO_4$	1.0
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.1
$CaCl_2$	0.1
Yeast Extract	1.0
Reese's Medium (pH=5.3) (medium R or TV medium)	
KH_2PO_4	2.0
$(NH_4)_2SO_4$	1.4
Urea	0.3
$MgSO_4 \cdot 7H_2O$	0.3
$CaCl_2$	0.3
Proteose Peptone	1.0
Trace Elements	1 ml/liter

Table II (cont.)

Trace Elements for Reese Medium	Concentration mg/100 ml distilled H_2O
$MnSO_4 \cdot H_2O$	156.0
$FeSO_4 \cdot 7H_2O$	500.0
$ZnCl_2$	167.0
$CoCl_2$	200.0
19% HCl	1 ml.

P Medium (pH=7.8)	Concentration g/l tap H_2O
$(NH_4)_2HPO_4$	10.0
K_2HPO_4	5.0
Na_2SO_4	0.5
Microelements #4 (Salts B)	10.0 ml.

Microelements #4 Salts B	Concentration gm/l distilled H_2O
$MgSO_4 \cdot 7H_2O$	40.0
$FeSO_4 \cdot 7H_2O$	2.0
$MnSO_4 \cdot 4H_2O$	1.5
NaCl	2.0

Medium RG

Reese's medium with glycine, 0.1%, instead of protopeptone

Medium RC

Reese's medium with casamino acids, 0.1%, (Difco Certified) instead of protopeptone.

Medium RY

Reese's medium with yeast autolysate, 0.1%, (Amber Labs, Inc., BYF Series 100) instead of protopeptone.

Medium F (pH 5.3)	Concentration, g/l tap H_2O
$(NH_4)_2SO_4$ (fertilizer grade)	10.0
$Na_2S_2O_3$	0.01

Medium G (pH 5.3)	Concentration, g/l tap H_2O
$(NH_4)_2HPO_4$ (C.P.)	10.0
$Na_2S_2O_3$	0.01

Medium H (pH 5.3)	Concentration, g/l tap H_2O
$(NH_4)_2SO_4$	5.0
$(NH_4)_2HPO_4$	5.0
$Na_2S_2O_3$	0.01

Medium G-1

Medium G + 0.03% urea

Medium G-2

Medium G + 1% $CaCO_3$

Medium G-3

Medium G + 0.03% urea + 0.1% yeast autolysate (Amber Labs, Inc., BYF Series 100)

Medium G-4

Medium G + 0.03% urea + 0.05% yeast autolysate (Amber Labs, Inc., BYF Series 100)

Medium G-5

Medium G + 4% extra $(NH_4)_2HPO_4$

Medium G-6

Medium G-1 + 4% extra $(NH_4)_2HPO_4$

Medium G-7

Medium G + 9% extra $(NH_4)_2HPO_4$

Medium G-8

Medium G-1 + 1.0 ml/l trace elements for medium G

Medium G-9

Medium G + 1.0 ml/l trace elements for medium G

Trace Elements for Medium G

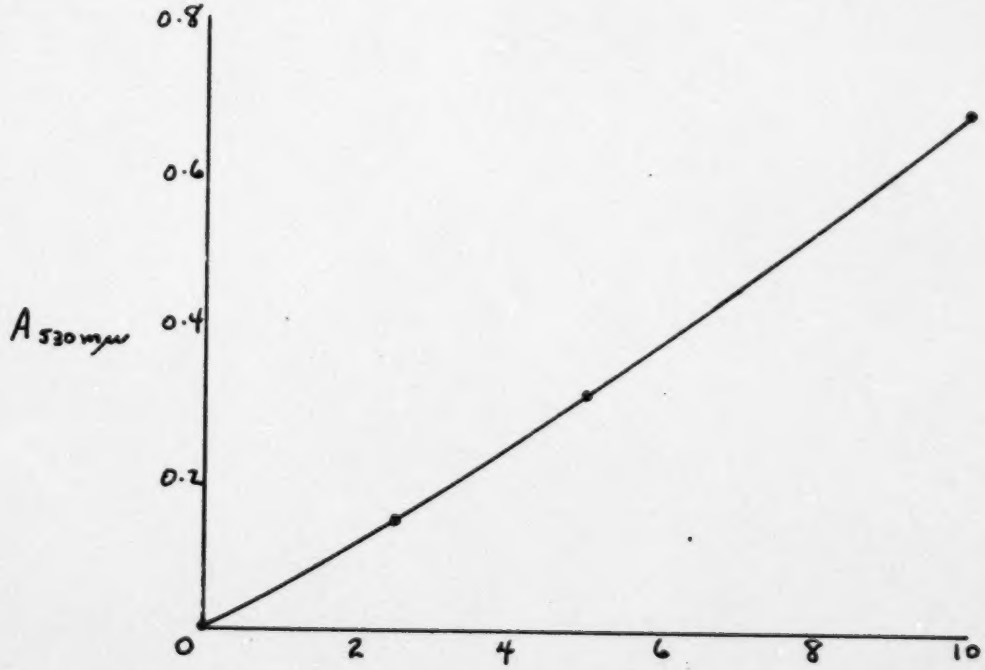
$CaCl_2$	30 g/l
$MgSO_4$	3.0 g/l
$CuSO_4 \cdot 5H_2O$	0.05 g/l

Use 1 ml/liter of media plus 1 ml trace elements for Medium R/liter media.

Original Biuret Protein [3, 16] Method

1. To 5.0 ml homogenized sample in a test tube add 1.0 ml 5 N NaOH. Mix well and allow to stand one hour.
2. Add 0.2 ml 20% copper sulfate. Mix well and allow to stand 30 minutes.
3. Filter through Whatman #1 paper and collect filtrate in another test tube.
4. Read absorbance at 530 mμ in 12 mm cuvettes versus a reagent blank of 5.0 ml distilled water carried through the procedure as above, steps 1-3.
5. Compare with a protein standard curve obtained similarly. A dilution of Versatol (Warner-Chilcott Labs) containing 5.0 mg protein/ml is a suitable standard. Use 0, 0.5, 1.0 and 2.0 ml standard diluted to 5.0 ml distilled water to correspond to 2.5, 5.0 and 10.0 mg protein/ 5 ml sample. Read the result directly from the curve in mg protein/ 5 ml sample.
6. 2.5 mg protein in 5 ml will produce an absorbance of about 0.30. Therefore, it is possible to detect about 0.1 mg protein per ml sample. The curve is essentially linear through 10 mg protein/5 ml sample.

STANDARD CURVE FOR ORIGINAL BIURET METHOD FOR DETERMINATION OF PROTEIN



SPECTRONIC-20
12 MM PATHLENGTH

A "blank" is run by the same procedure on the freshly inoculated culture medium which was used in the fermentation in order to obtain the initial protein concentration of the medium. The final figure for protein produced was derived by subtracting this value from the protein value of the fermentation beer.

Revised Biuret Protein Method [15]

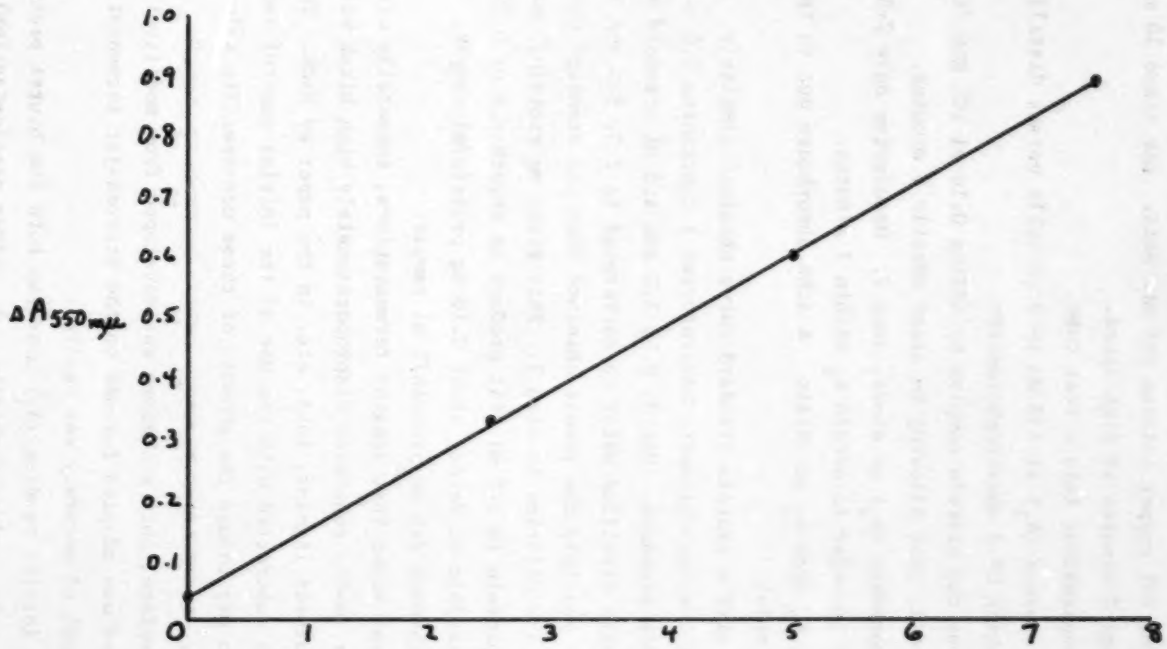
1. To 5.0 ml sample in a 15 ml centrifuge tube, add 5.0 ml distilled water and 2.0 ml 5 N NaOH. Mix well by inversion. Let stand one hour.
2. Centrifuge 5 minutes at high speed in clinical type centrifuge.
3. Transfer 6.0 ml supernatant to another 15 ml centrifuge tube.
4. Add 0.2 ml 20% copper sulfate and mix well. Let stand 30 minutes.
5. Centrifuge 5 minutes at high speed.
6. Decant supernatant into a test tube.
7. Read absorbance (A_1) at 550 mμ in 2 cm cells versus distilled water on a Beckman DK-2 spectrophotometer.
8. Decolorize the protein complex by adding 0.10 ml 30% KCN (caution - use hood) mixing well, then allowing to stand exactly 5 minutes.
9. Read absorbance (A_2) as above, step 7. Decolorize only 2-3 samples at one time in order to obtain A_2 within 7 minutes.
10. Subtract A_2 from A_1 to obtain A (the absorbance due to the protein complex only).
11. Compare with a protein standard curve obtained similarly. A dilution of Versatol (Warner-Chilcott Laboratories) containing 5.0 mg protein/ml is a suitable standard. Use 0, 0.5, 1.0 and 1.5 ml standard diluted to 5.0 ml with distilled water to correspond to 2.5, 5.0 and 7.5 mg protein/5 ml sample. Multiply the result obtained from the standard curve by 2 to correct for the 1:2 dilution in step 1. This gives mg protein/5 ml sample.
12. 2.5 mg protein in 5.0 ml will produce an absorbance of 0.28-0.31. Therefore, it is possible to detect about 0.10 mg protein/ml sample. The curve is linear through 7.5 mg protein/5 ml sample.

It was shown that lengthy fermentations, expecially with high concentrations of newspaper (4-8%) generated disproportionately high blank values due to extraction of highly colored lignins, inks, etc. in the paper by NaOH. These could not be legitimately subtracted with the use of the initial control samples, since the fermentation may change the amounts of these non-specific colors as well as the biuret color.

Therefore, this procedure was developed from modifications of existing methods[15] and was adopted for use on the stirred-jar fermentation runs 6-12 where a higher level of accuracy was desired.

The initial reading (A_1) measures both the biuret protein complex and interfering color and/or turbidity. A_2 (after decolorization) measures all interferences which are then subtracted to obtain the absorbance due to protein only.

Standard Curve for Revised Biuret Method for Determination of Protein



Beckman DK-2
2.0 cm pathlength

Calculation of Corrected Protein and Cellulose Values

A. Shake-Flasks

To correct for evaporation losses which occurred during autoclaving and fermentation, all flasks were diluted to their original volume of 100 ml, then sampled and analyzed for protein and cellulose content.

The protein produced, or cellulose utilized was calculated by obtaining the difference from uninoculated controls.

Example: Data from Table XXV

Flask No.	Uncorrected Protein Present mg/ml	Corrected Protein Produced mg/ml
8	0.91	0.57
10	0.84	0.50
12	0.88	0.54
14(Control)	0.34	-

B. Stirred-Jar Fermentors

The actual protein and cellulose content of the stirred jar fermentations at a given moment were influenced by such factors as evaporation (concentrative effect), which increased with the use of higher aeration rates, and the addition of antifoamant (diluent effect), if any.

Volumes removed for analyses were measured in order to determine evaporation losses, but no correction was applied since these were a homogeneous blend of the total volume present.

Thus, in order to relate the values back to the original volume, correction factors were derived and applied to each case by the following method:

1. evaporation/day was computed,
2. the difference between the evaporation and the amount of antifoamant added was found,
3. this difference (+ or -) was subtracted from (or added to) the initial volume which gives the volume at that time.
4. volume at that time/initial volume=correction factor
5. (correction factor)(protein or cellulose content)= corrected protein or cellulose content.

Example: Fermentor #1 in experiment #12

1. Evaporation in 12 days = 610 ml
2. Antifoamant added in 12 days = 480 ml;
   difference = -130 ml
3. Initial volume = 7530 ml;
   7530 - 130 = 7400 ml
4. Correction factor = 7400/7530 = 0.983
5.

Day	Uncorrected Protein Produced, mg/ml	Uncorrected Cellulose Remaining, mg/ml	Correction Factor	Corrected Protein Produced, mg/ml	Corrected Cellulose Remaining, mg/ml
12	1.44	7.88	0.983	1.42	7.75

μσ652